Characterization of Semaphorin 6A during Development of the Embryonic Chicken Spinal Cord

Dissertation
zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)
vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich
von

Rejina Sadhu
aus
Indien

Promotionskomitee

Prof. Dr. Esther Stoeckli (Leitung der Dissertation)
Prof. Dr. Alex Hajnal
Dr. Matthias Gesemann

Zürich, March 2006

Zusammenfassung

Für die Bildung des informationsverarbeitenden Netzwerks, das unser Nervensystem darstellt, müssen Nervenzellen während der Embryonalentwicklung mit grosser Präzision verknüpft werden. Während der letzten 20 Jahre wurden die Prinzipien der axonalen Wegfindung erarbeitet. Insbesondere wurde eine Vielzahl von Wegweisermolekülen entdeckt, die für die axonale Wegfindung von entscheidender Bedeutung sind. Eine Familie von Wegweisermolekülen sind die Semaphorine. Sie wurden ursprünglich als repulsive Moleküle identifiziert, die axonale Wegfindung beeinflussen. Semaphorine kommen in löslicher und membrangebundener Form vor. In letzter Zeit wurden auch weitere Funktionen von Semaphorinen beschrieben, wie z.B. axonale Faszikulation, Auswahl der Zielzellen, Zellwanderung und Wegfindung von Dendriten. Ausserdem wurden Semaphorine auch mit Plastizität und Regeneration im adulten Nervensystem in Verbindung gebracht. Als Rezeptoren von Semaphorinen wurden Plexine und Neuropiline identifiziert.

In meiner Doktorarbeit habe ich die Funktion des transmembranalen Semaphorins 6A während der Entwicklung des Nervensystems untersucht. Semaphorin6A ist in den sog. Boundary Cap Zellen exprimiert. Das Blockieren der Funktion von Semaphorin6A mittels *in ovo* RNAi (*in ovo* RNA Interferenz) führte zu einer fehlerhaften Anordnung der Axonbündel, die von den Spinalganglien ins Rückenmark wachsen. Motoneuronen, die normalerweise im ventralen Teil des Rückenmarks liegen, wurden ausserhalb des Rückenmarks entlang der Vorderwurzeln entdeckt. Diese Beobachtungen bestätigen somit die Hypothese, dass Semaphorin6A in den Boundary Cap Zellen als “Gate Keeper” Eintritt und Austritt aus dem Rückenmark kontrollieren.

Summary

Neuronal connections are made during embryonic development with astonishing precision to ultimately form the information processing network of the nervous system. Over the past few decades, much has been learned about the general principles of axon guidance. Many molecular cues have been discovered which help establishing these connections accurately. One family of axon guidance cues, the semaphorins, was first identified as repellents for navigating axons during brain wiring. Semaphorins are secreted, membrane-attached or transmembrane in nature. Recent studies have implicated these molecules in many other processes of neuronal development, including axonal fasciculation, target selection, neuronal migration, and dendritic guidance, as well as in the remodeling and repair of the adult nervous system. The functions of Semaphorins are mediated by receptor complexes consisting of Plexins and Neuropilins. In my thesis, I characterized the function of a transmembrane semaphorin, SEMA6A, in the developing chicken spinal cord. Chicken SEMA6A is expressed in boundary cap cells. Silencing SEMA6A by *in ovo* RNAi (*in ovo* RNA interference) led to aberrant arrangement of the dorsal roots and emigration of motor neurons from the spinal cord. Thus, these data suggest a role for SEMA6A as a gatekeeper at the CNS/PNS interface.

Contents

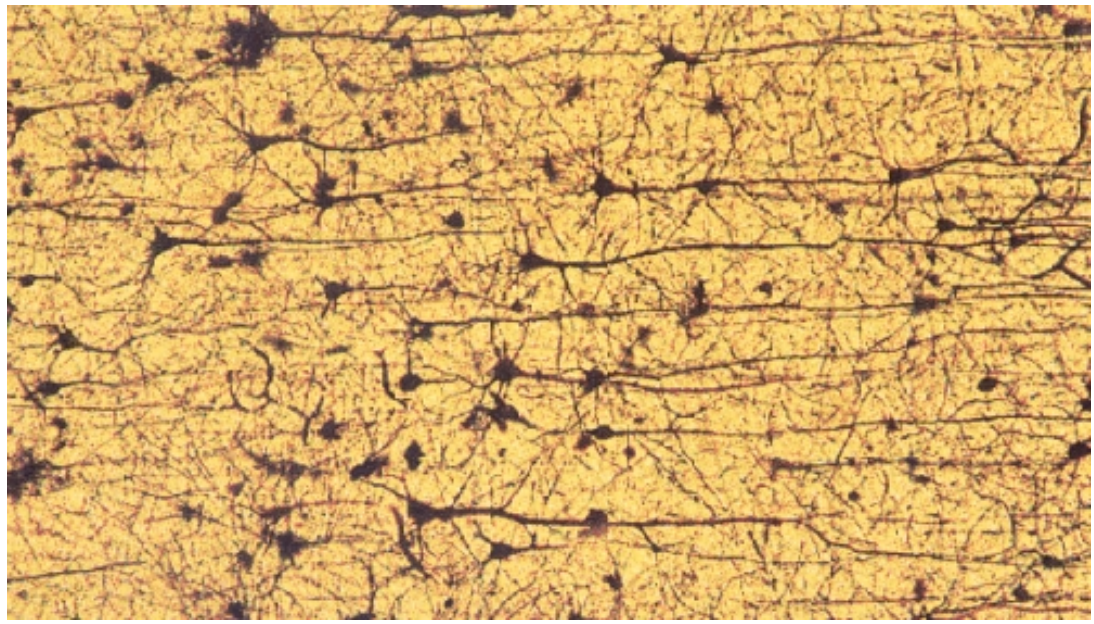
Zusammenfasung

Summary

Chapter 1

Introduction

1.1 Introduction-Organization of the nervous system



Neuronal network (Ramon y Cajal)

1) Finding the right target: Axon guidance and pathfinding

The function of the nervous system depends on a precise and elaborate network of connections among neurons. Every action and reaction executed in one's lifetime involves communication between a large number of neurons and their targets. The efficiency of this complex process depends upon precision in neuronal connectivity.
How is this connectivity established?

- The growth cone and the mechanism of axon guidance:
  - For the nervous system to function properly, all the myriad neurons need to connect accurately to one another as well as to peripheral targets. To make these connections, each developing nerve cell extends a long process, the axon, which grows towards the target cells. At the tip of the growing axon is a specialized structure called growth cone. The role of the growth cone is to navigate through a dense maze of different cells that lie between the neuronal cell body and the target cells by sensing and integrating molecular cues presented on, or secreted by surrounding cells (Fig 1.1).

  A century ago, Ramón y Cajal first proposed the existence of the growth cone based on his morphological observations. He also suggested the existence of chemoattraction. Later on, based on experiments with cultured neurons from chick embryos, the concept of chemorepulsion was added. When axons are extending through a permissive substrate, the growth cone has a very simple, streamlined morphology with few filopodial projections. However, when the growth cone encounters a decision or choice point, i.e. a place where an axon might turn, the growth cone acquires a complex morphology that is characterized by extension of lamellipodial webs, together with projection of multiple filopodia. The growth cone also slows down allowing it to integrate the new guidance information that it has just encountered at the choice point. As the axon extends beyond the choice point, the growth cone once more adopts a simple appearance

until it reaches the next decision point, or the ultimate target region. Once within the target region, the growth cone becomes even more elaborate, by arborizing to form branched terminals, as it changes shape to establish connections with postsynaptic targets.

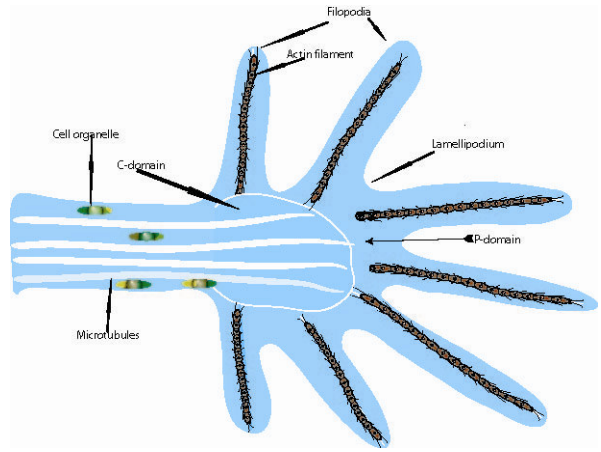


Fig: 1.1: The growth cone.

The growth cone has several finger-like protrusions at the tip known as filopodia which are interspersed with web-like structures known as lamellipodia. Using the filopodia the growth cone senses the immediate environment.

2) Axon guidance molecules:

A plethora of molecules have been described which contribute to axon guidance. These can be broadly classified into the following families of guidance cues, which provide directional information to growing axons: the Immunoglobulin superfamily of cell adhesion molecules (IgCAMs), the ephrins, the netrins, the slit proteins and the semaphorins (Figs: 1.2 & 1.3).

- IgCAMs
  - IgCAMs are a large, ubiquitous, but fairly conserved family of surface proteins. The characteristic extracellular immunoglobulin-like domain involves one or more folds of 60 to 100 amino acids. IgCAM interactions can be either homophilic or heterophilic depending upon the type of cell adhesion molecule. These molecules have been studied intensively in the context of neuronal development in

vertebrates (Burden-Gulley et al., 1995; Rutishauser, 2000; Yu and Bargmann, 2001). *In vitro* assays have been used to demonstrate their capacity to promote neurite outgrowth and their role in fasciculation. Especially intriguing is the complex interaction pattern of these molecules (Brummendorf et al., 1993; Brummendorf and Rathjen, 1993; Brummendorf and Rathjen, 1995; Brummendorf and Rathjen, 1996; Sonderegger and Rathjen, 1992). Lynn Landmesser and colleagues described the role of Ig superfamily CAMs quite extensively during the innervation of the chicken hindlimb (Landmesser et al., 1988; Landmesser et al., 1990; Tang et al., 1992; Tang et al., 1994).

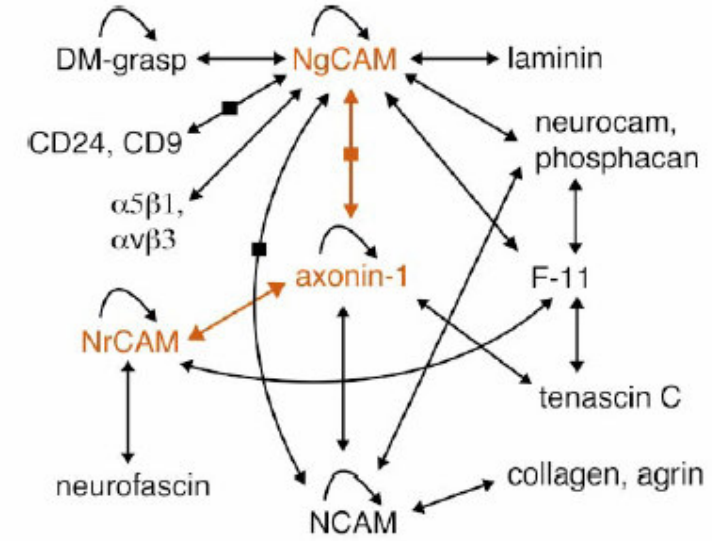


Fig: 1.2: Summary of molecular interactions involving Ig super-family CAMs found on vertebrate axons. Looped single arrows represent homophilic binding. Double-headed arrows represent heterophilic binding; when a square is also present on the arrow, the binding occurs within the plane of the same membrane (cis). (Adapted from (Brummendorf et al., 1998; Rutishauser, 2000).

These studies have elucidated the importance of the polysialic acid component of NCAM and its impact on NgCAM-mediated fasciculation for correctly sorting motoneuron fibers in the plexus region. Older *in vivo* studies were aimed at investigating the role of NCAM in the development of the retino-tectal system (Silver and Rutishauser, 1984; Thanos et al., 1984). Stoeckli and Landmesser suggested the involvement of Axonin-1, NgCAM and NrCAM as pathfinding molecules

for commissural neurons in the chicken spinal cord (Stoeckli and Landmesser, 1995). They suggested that interactions between Axonin-1 and NrCAM induce commissural growth cones to enter the floorplate and in their absence, the floorplate was less attractive or even inhibitory. NgCAM on the other hand seemed to be more important in maintaining the fasciculation of commissural neurons rather than directionality. Later on, experiments using a two-dimensional coculture system of commissural and floor-plate explants showed that Axonin-1 and NrCAM were crucial for the interaction between commissural growth cones and the floorplate, which in turn was required for the proper guidance of axons across the ventral midline and their subsequent rostral turn into the longitudinal axis (Stoeckli, 1998; Stoeckli and Landmesser, 1998; Stoeckli et al., 1997).

- Ephs and Ephrins:
  - Ephs are family of receptor tyrosine kinases (RTKs) (Shao et al., 1994; Shao et al., 1995; Tessier-Lavigne, 1995) and the Ephrins are their membrane-bound ligands (Beckmann et al., 1994; Brambilla et al., 1995; Drescher et al., 1995; Kozlosky et al., 1995). The Ephs and Ephrins have been implicated in many events like cell migration, somitogenesis, angiogenesis, axon pathfinding (Davy and Soriano, 2005) e.g. commissural axon pathfinding (Fig 1.4) (Imondi and Kaprielian, 2001; Palmer and Klein, 2003) and also processes like oocyte maturation (Hall et al., 1999) and cerebellar granule cell migration (Lu et al., 2004; Lu et al., 2001).

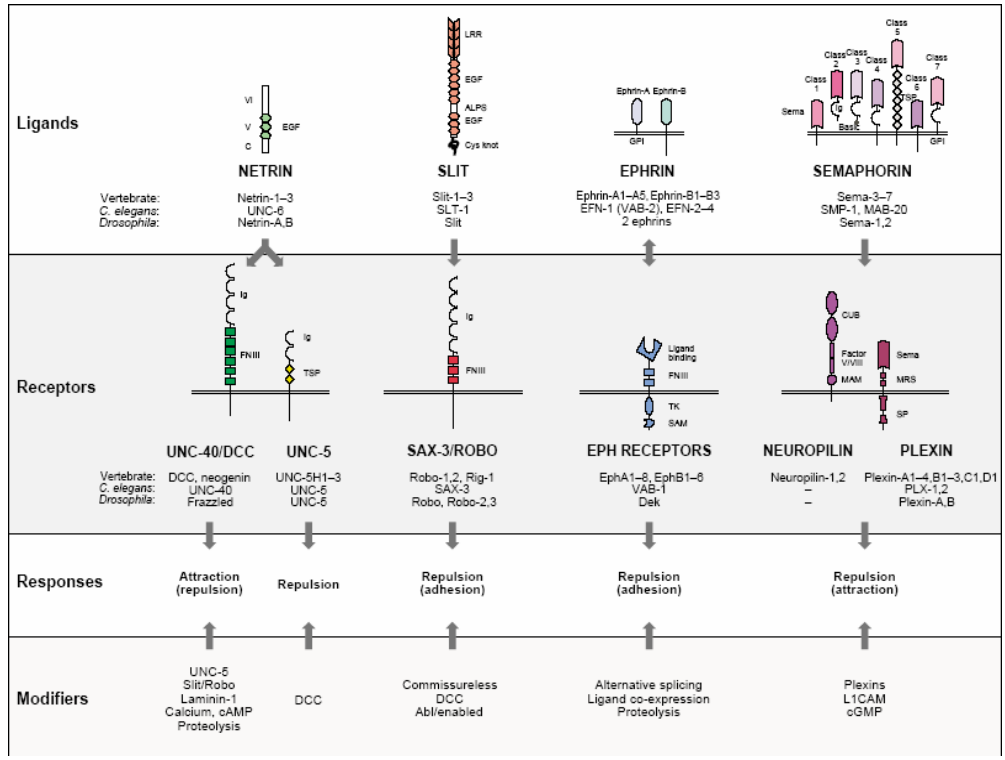


Fig: 1.3: Overview over the four main families of instructive guidance cues and their receptors. Major families are indicated by column headings, with species-specific names underneath. Typical guidance responses, alternate responses (in parentheses) and known modifiers of each pathway are shown. ALPS, agrin–laminin–perlecan–Slit domain; C, netrin C terminus; CUB, C1/Uegf/BMP-1 domain; DCC, deleted in colorectal cancer; EGF, epidermal growth factor; FNIII, fibronectin type III domain; GPI, glycosylphosphatidyl–inositol anchor; Ig, immunoglobulin domain; LRR, leucine-rich repeat; MAM, meprin/A5 antigen motif; MRS, Met tyrosine kinase–related sequence; RK, arginine/lysine-rich basic domain; SAM, sterile alpha motif; SP, 'sex and plexins' domain; TK, tyrosine kinase domain; TSP, thrombospondin domain; VI and V, homology to laminin domains VI and V, respectively (Yu and Bargmann, 2001).

- Receptor–ligand pairings are promiscuous within a subgroup (A or B) but there is rarely any intergroup binding. Receptors and ligands form complementary expression gradients in various parts of the nervous system (Cheng and Flanagan, 1994; Cheng et al., 1995). Ephs and Ephrins are subdivided into two classes: Ephrin-As, which are anchored to the membrane via GPI linkage and preferentially bind EphA receptors, and Ephrin-Bs, which are transmembrane proteins that preferentially interact with receptors of the EphB subtype. The only

known exception is the EphA4 receptor, which has been shown to interact with members of both class A and class B Ephrins (Brors et al., 2003; Kullander et al., 2003; Kullander et al., 2001a). Generally, cells expressing Eph receptors avoid territories expressing Ephrins, thus providing necessary cues to guide axons to their appropriate target (O'Leary and McLaughlin, 2005; O'Leary and Wilkinson, 1999). However, It has been recognized

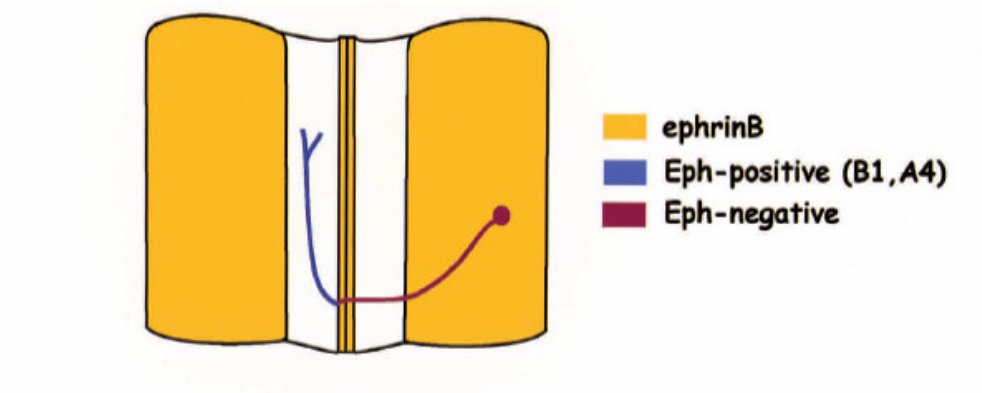


Fig: 1.4: Schematic drawing of an "open-book" preparation of an embryonic chick spinal cord (anterior up, posterior down) with ephrinB ligands expressed by the floorplate (in the *middle*) and by the dorsal to intermediate parts of the spinal cord (in yellow). Commissural neuron cell bodies are located in the dorsal spinal cord (in purple). Pre-crossing axons of commissural neurons are Eph-negative (in purple) and therefore do not respond to Ephrins. Postcrossing distal axon segments up-regulate EphB1 and EphA2 expression (in blue) and are confined into a narrow longitudinal path by ephrinB ligands (Palmer and Klein, 2003).

more recently that Eph receptors and Ephrins can also regulate axon pathfinding through attractive interactions (Eberhart et al., 2004; Hindges et al., 2002; Knoll et al., 2001; Kullander et al., 2001b; Mann et al., 2002; Palmer and Klein, 2003). Anterior–posterior (A–P) topographic targeting of retinal axons is controlled by a graded distribution of EphAs and EphrinAs in retina and midbrain, respectively (Flanagan and Vanderhaeghen, 1998; Klein, 2001; O'Leary and McLaughlin, 2005; O'Leary and Wilkinson, 1999; Wilkinson, 2000). Ephs and Ephrins were recognized early on for their role in segmentation (Wilkinson, 2000) and later on were also found to

regulate both cranial and trunk neural crest cell (NCC) migration (Holder and Klein, 1999; Wilkinson, 2000). Motor neuron innervation of limb muscles is also in part regulated by ephrinA/ EphA interactions (Eberhart et al., 2004; Eberhart et al., 2000; Eberhart et al., 2002; Feng et al., 2000; Klein, 2001).

- Netrins:
  - The Netrins are a small family of phylogenetically conserved secreted proteins with amino acid sequence similarity to proteins of the laminin family (Livesey, 1999; Livesey and Hunt, 1997). Four vertebrate members of this family have been identified till now: Netrin-1 to Netrin-4 (Yin et al., 2000). Netrins are secreted from the floorplate and ventral spinal cord and act as a chemoattractant for commissural axons (Fig 1.5) (Cooper et al., 1999). *In vitro* studies have shown that Netrins can also act as chemorepulsive agents for trochlear motor axons (Colamarino and Tessier-Lavigne, 1995a). However, there are no obvious defects in the pathway taken by these motor axons in mice lacking netrin. These two examples suggest the bifunctionality of Netrins- both attractive and repulsive activity. Remarkably, localized protein synthesis within the neuronal growth cone is required for Netrin-1-induced attraction and repulsion (Campbell and Holt, 2001).The function of netrins has been evolutionarily conserved in a remarkable way; orthologs with similar functions were first identified in *C. elegans* (UNC-6)(Ishii et al., 1992; Kennedy et al., 1994; Serafini et al., 1994) and have also been identified in *Drosophila* (Harris et al., 1996; Mitchell et al., 1996). The putative receptors for UNC-6 and *Drosophila* netrins are UNC-40 (Chan et al., 1996) and frazzled (Kolodziej et al., 1996). Based on structural analyses these molecules can be considered as orthologs of DCC, the receptor for Netrin identified in mammals (Keino-Masu et al., 1996).

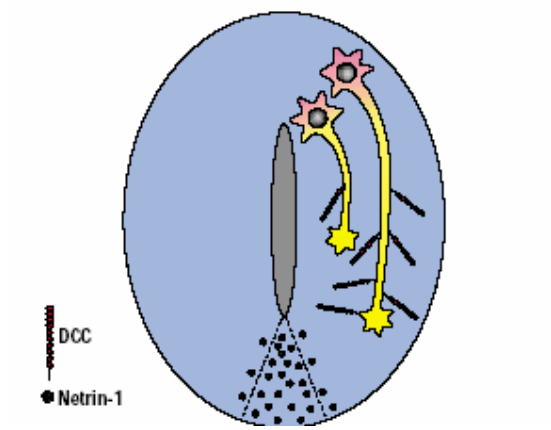


Fig.1.5: Netrin interacts with its receptor DCC to attract commissural axons toward the ventral midline

(Cooper, 2002)

- Slits:
  - Slit proteins are large (~190 kDa) extracellular matrix molecules containing leucine-rich repeats and epidermal growth factor (EGF)-like repeats. They are secreted proteins that signal through receptors of the Roundabout (Robo) family. Robo was first identified in a genetic screen for midline guidance defects in Drosophila (Kidd et al., 1998a; Seeger et al., 1993). Genetic studies suggested that Robo is the receptor for a midline repellent (Kidd et al., 1998b; Stoeckli and Landmesser, 1998)), subsequently identified as Slit (Battye et al., 2001; Kidd et al., 1999). The Robo-Slit interaction is conserved in vertebrates (Brose et al., 1999; Li et al., 1999). The Robo-Slit interaction has been implicated in commissural guidance axon guidance across the midline (Fig 1.6) (Long et al., 2004; Rajagopalan et al., 2000; Stoeckli and Landmesser, 1998).

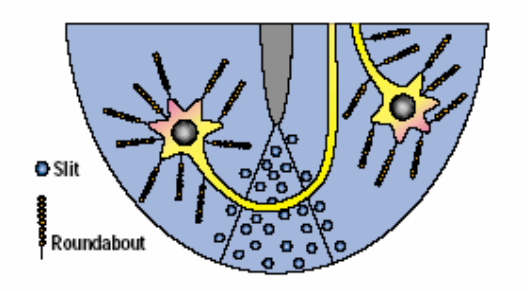


Fig: 1.6: The interaction between Robo and Slit plays a significant role in midline crossing of commissural axons. The chemorepulsive guidance cue, Slit, is expressed by the floorplate. It is proposed that the Slit receptor, Robo, is expressed at high levels on those axons that never cross the midline. Axons destined to cross the midline express very low levels of Robo when projecting on the ipsilateral side. Once on the contralateral side, Robo protein is up-regulated on the axonal membrane and these axons never cross the midline again (Cooper, 2002).

- Semaphorins:
    i. An Overview:

The semaphorins represent one of the largest families of axon guidance cues consisting of eight classes (Fig 1.7). Members are classified according to structural criteria. All semaphorins share a highly conserved Sema-domain of about 500 amino acids (Tamagnone et al., 1999) with 17 conserved cysteines. Classes 1 and 2 are found only in invertebrates, while classes 3 to 7 contain the vertebrate family members, and class V members are encoded by viral genomes. Semaphorins include both transmembrane (classes 1, 4, 5 and 6), glycosylphosphatidyl inositol (GPI; class 7) and secreted (classes 2, 3 and V) proteins (Kolodkin et al., 1993). Semaphorins have been implicated in various processes for example, in development of bone (Togari et al., 2000) and vasculature (Shima and Mailhos, 2000), cancer metastasis (Xiang et al., 1996), B-cell aggregation and differentiation (Hall et al., 1996) as well as in inhibiting synaptic terminal arborization (Matthes et al., 1995), and axon guidance (Messersmith et al., 1995; Puschel et al., 1995; Puschel et al., 1996; Wright et al., 1995). Semaphorins were first suggested as putative axon guidance molecules due to the growth cone collapsing activity of Sema3A/Collapsin (Luo et al., 1993). Initially, all semaphorin family members were believed to induce inhibitory actions on axon pathfinding, branching or targeting, but there is increasing evidence that semaphorins may also have a role in chemoattraction (de Castro et al., 1999; Kolodkin et al., 1992; Luo et al., 1993; Matthes et al., 1995; Messersmith et al., 1995).

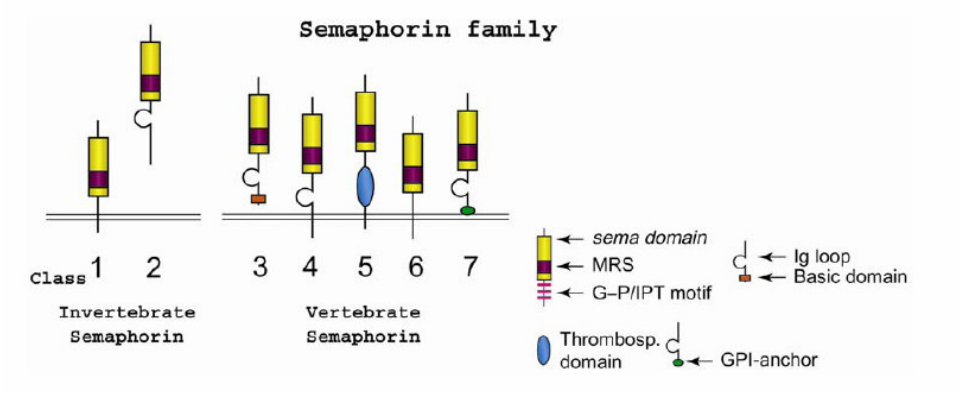


Fig: 1.7: Semaphorins are subdivided into different classes based on structural criteria. Semaphorins are phylogenetically related proteins, sharing sema domains similar to the cysteine rich Met-related sequences (MRSs). Semaphorins are divided into 7 subgroups excluding the viral semaphorins. Class 1 & 2 exist only in invertebrates whereas Classes 3-7 are found in vertebrates. Class 2&3 are secreted semaphorins while all the other members are membrane bound; Class 1, 4, 5 and 6 are transmembrane and Class 7 contains GPI-linked members. Abbreviations: G-P/ IPT motif: glycine-proline repeat/ immunoglobulin-like fold; GPI, glycosyl phosphatidylinositol; MRS, Met-related sequence (Tamagnone and Comoglio, 2000).

ii. Secreted semaphorins- Versatile in their expression and function

Sema II is the only secreted semaphorin in invertebrates. It is expressed transiently in a subset of motor neuron during motor neuron outgrowth and synapse formation. Gain-of-function experiments carried out in Drosophila show that ectopic Sema II expression in muscle cells inhibits normal synaptic terminal arborization of two different motor neurons subtypes without affecting the growth cones of other motor axons. The fact that ectopic Sema II expression does not affect the oriented growth of axons toward these muscles suggests that Sema II serves as a selective target-derived signal inhibiting the formation of specific synaptic terminals, rather than influencing early aspects of axon guidance (Matthes et al., 1995).

The class 3 semaphorins are the most extensively studied class of semaphorins. They are the only vertebrate class of secreted

semaphorins. These molecules were first called collapsins and characterized for their ability to collapse the growth cones of chick dorsal root ganglia (Luo et al., 1993; Raper and Kapfhammer, 1990). They were identified by two independent approaches. Luo and colleges identified vertebrate Sema3A from the brain and due to its collapsing effect on axons called it collapsin (Luo et al., 1993) and Raper's group identified Sema3A as the major growth inhibitory protein for sensory axons (Luo et al., 1993). The members of this class share three structural motifs, the 500 amino acid sema domain, a C-2 type immunoglobulin (Ig) domain, and a positively charged carboxy terminal tail. Secreted semaphorins act as diffusible signals, although their diffusion distance might be limited because the charged sequence at the C-terminus of the protein makes them stick to cell surfaces and extracellular matrix *in vivo* (Bagnard et al., 2000).

Sema3A seems to be crucial during development for directing the sensory projections of the dorsal root ganglia (DRG) neurons in the spinal cord and to the skin (Shepherd et al., 1996). Sema3A was reported to affect development of the perforant pathway of the entorhinal hippocampal formation (Chedotal et al., 1998; Steup et al., 1999), pontocerebellar mossy fiber projections (Rabacchi et al., 1999), the patterning of cortical efferents (Bagnard et al., 1998; Polleux et al., 1998), suppression of migration of avian trunk neural crest cells (Eickholt et al., 1999) and directing cranial nerve migration (Taniguchi et al., 1997). In spite of its wide expression in the developing central and peripheral nervous systems, Sema3A is very specific in its actions. In dorsal root ganglia, only NGF-sensitive sensory axons are affected by Sema3A while NT3-sensitive axons are not affected (Messersmith et al., 1995). It collapses axons of sympathetic ganglia neurons (Puschel et al., 1996), motor neurons (Shepherd et al., 1996; Varela-Echavarria et al., 1997), sensory neurons from the trigeminal, facial and vagal cranial ganglia, in addition to axons from olfactory neurons (Kobayashi et al., 1997). In the adult, Sema3A expression diminishes greatly and is restricted to areas where it inhibits new connections. A

sustained decline in sema3A/coll-1 mRNA expression was found when regeneration was blocked by nerve transection and ligation. This observation has led to the belief that it is involved in CNS nerve regeneration. Taken together, the temporal and spatial expression pattern of sema3A/coll-1 mRNA and its relationship to emerging nerve tracts suggests that it is involved in guiding growing axons towards their targets by forming a molecular boundary that instructs axons to engage in the formation of specific nerve tracts (Giger et al., 1996; Pasterkamp et al., 1998). Mice homozygous for a targeted mutation in semaphorin3A show severe abnormality in peripheral nerve projection. Aberrations are seen in the trigeminal, facial, vagus, accessory, and glossopharyngeal nerves but not in the occulomotor nerve. These results suggest that semaphorin3A functions as a selective repellent *in vivo* (Taniguchi et al., 1997) and is important as an axon guidance cue for peripheral projections (Ulupinar et al., 1999). However, the misprojections in embryonic Sema3A knock-out mice are all corrected or eliminated by E15.5 suggesting late-embryonic expression of a redundant repulsive semaphorin or other guidance cue that correctly prunes the aberrant arborization (White and Behar, 2000). Different studies conducted with regard to Sema3A disruption or knockouts have resulted in conflicting observations.

Initially five different avian class 3 semaphorins were characterized (Luo et al., 1995). Recent, extensive databank searches using the combined information from the EST and the genomic database to predict the number of chicken semaphorin genes by Joelle Gemayel and Matthias Gesemann has revealed the presence of an additional class 3 semaphorin member referred to as Sema3G. Phylogenetic analysis of the conserved semaphorin domain revealed that the novel Sema3G is most closely related to Sema3E. However, the conservation of the sema domain of chicken Sema3G and mouse Sema3G is the lowest between all class 3 semaphorins (Gemayel et al, unpublished). This novel member was to be mapped on chromosome 14 in mice. In adulthood, Sema3G is mainly expressed in the lung and

kidney, and at low levels in the brain. In the adult rodent brain, it is expressed only in the granular layer of the cerebellum. Sema3G binds Neuropilin-2, but not Neuropilin-1, and induces the repulsion of sympathetic axons, but not dorsal root ganglion axons (Taniguchi et al., 2005). In zebrafish, Sema3F, Sema3G and Npn2 signaling normally contributes to the guidance of migrating cranial neural crest cells (Yu and Moens, 2005).

Other members of class 3 semaphorins have been analyzed less well than Sema3A. *In vitro* experiments demonstrate that Sema3B can repel sympathetic axons (Takahashi et al., 1998) but not much is known about the role of Sema3D and Sema3E (Koppel et al., 1997; Raper, 2000). Recently, it was shown that sonic hedgehog was important for correct expression of Sema3D and slits (Barresi et al., 2005). Sema3D is implicated in retinal axon pathfinding in zebrafish (Liu and Halloran, 2005; Sakai and Halloran, 2006) while Sema3E has been shown to interact with plexinD1 in controlling vascular patterning (Yu and Moens, 2005). The mRNA expression for five members of the class-3 semaphorin family (3A, 3B, 3C, 3E and 3F) was seen in adult retinal ganglionic cells (RGCs). Expression was highest in DRGs for semaphorins 3B and 3C, and lowest for 3A. Levels of mRNA expression in RGCs were lower in newborn retinas but were raised by P14. Expression by different cell types in the inner nuclear layer was also seen, especially at P14 suggesting a potential role for these proteins in retinal development and in the maturation, stabilization, and plasticity of mammalian primary visual pathways (de Winter et al., 2004). The spatiotemporal regulation of Sema3B and Sema3F in the brainstem and developing head, including the eye, ear, and branchial arches provide a basis for functional analysis of these molecules in the development of axon projections and the morphogenesis of cranial structures (Chilton and Guthrie, 2003).*In vitro* Sema3C repels neurites from CA1 and medial septum but has no effect on CA3, dentate gyrus and entorhinal axons. Interestingly Sema3C appears to exert a dual role *in vitro* depending on the neuronal population studied. It acts as a

repellent on sympathetic axons, has no effect on DRG neurons but in contrary attracts axons of cortical explants (Bagnard et al., 2000). Sema3C mutant mice show no obvious defects in the development of the nervous system. However, mutant mice display severe congenital cardiovascular defects and die soon after birth because of the interruption of the aortic arch and improper septation of the cardiac outflow tract. This phenotype is consistent with the expression of Sema3C in the mesenchyme surrounding the branchial arch arteries and in the myocardial cuff as well as the cardiac outflow tracts suggesting a role for Sema3C in guiding migratory cardiac crest cells. Moreover, this phenotype is similar to the phenotype observed on ablating cardiac neural crest cells in chick embryos and in humans with congenital heart diseases (Feiner et al., 2001).

Sema 3F was first discovered based on its expression in various cancer cells (Bielenberg et al., 2004; Xiang et al., 1996). It has subsequently been shown to affect pathfinding in CNS and PNS *in vitro* and *in vivo*. It is expressed in embryonic hippocampal regions in mice at the time of axonal outgrowth (E15 to E17) and shows repulsive activity on CA1, CA3 and dentate gyrus axons *in vitro* (Chedotal et al., 1998). SEMA3F transcripts were also localized along the caudal margin of the midbrain. Misexpression of SEMA3F demonstrated that Sema3F displays repulsive activity *in vivo* that guides the trochlear motor axons along the midbrain-hindbrain boundary (MHB). Trochlear motor axons project dorsally along the MHB to decussate at the dorsal midline (Watanabe et al., 2004); (Giger et al., 2000). Interestingly *in vivo* studies, based on the generation of Sema3F mutant mice, demonstrate that Sema3F is crucial for axon fasciculation and segregation but not for target recognition in the olfactory system (Cloutier et al., 2004). Additional analyses of the CNS of Sema3F mutant mice reveal that this protein is essential in the ventral forebrain for anterior commissure axons to fasciculate and decussate normally at the CNS midline as well for the formation of the infrapyramidal tract (Sahay et al., 2003). Mice lacking Sema3F are prone to seizures which

became explicit, when Sema3F application to acute hippocampal slices modulated both the frequency and the amplitude of miniature excitatory post-synaptic currents (EPSCs) in granule cells of the dentate gyrus and pyramidal neurons of CA1. These results suggest a novel role for semaphorins as synaptic modulators (Sahay et al., 2005). Moreover, it is also implicated in nerve regeneration modules as high levels of Sema3F mRNA is found below the injury site in the epi- and perineurium, in a sciatic nerve crush model, in which axonal regeneration is robust. Sema3F mRNA levels increase in peripheral nerves distal to a transection or crush injury (Scarlato et al., 2003).

The various effects of class 3 semaphorins have been summarized in the following table: **(Table 1.1).**

iii. Transmembrane or membrane-attached semaphorins- Dynamic regulation and diversity in function

- Transmembrane and membrane-attached semaphorins

Sema1 (Fasciculin IV), the first semaphorin member to be characterized, is a transmembrane protein expressed on subsets of fasciculating axons and clusters of epithelial cells in the grasshopper limb bud (Kolodkin et al., 1992). Ectopic expression experiments in grasshopper demonstrated that Sema1 played a crucial role in steering a pair of sensory neurons from the limb bud by regulating axon defasciculation and branching (Tessier-Lavigne and Goodman, 1996).

Reported activities for class 3 semaphorins in *in vitro* assays.	
Semaphorin Effect on axons	
SEMA-3A	Repels DRG, trigeminal (V), facial (VII), vagal (X), olfactory sensory, sympathetic , cortical, hippocampal , motor, cerebellar mossy
SEMA-3B	Repels sympathetic
SEMA-3C	Repels sympathetic Attracts cortical
SEMA-3D	None reported
SEMA-3E	None reported
SEMA-3F	Repels sympathetic Repels hippocampal Attracts olfactory bulb
SEMA-3G	Repels sympathetic but not DRG

Table: 1.1: The table summarizes results from a number of studies in which recombinant semaphorins were shown to either collapse or repel axons extending from cultured explants.

DRG: dorsal root ganglion. (Raper, 2000; Taniguchi et al., 2005).

Semaphorins belonging to subclass 4 and 6 have been studied mainly in the immune system where they exert immuno-modulatory effects. Sema4D/CD100, which is expressed constitutively by T cells, is involved in the activation of B cells and dendritic cells, while Sema4A is preferentially expressed on B cells and dendritic cells, and is involved in the activation of T cells (Kumanogoh and Kikutani, 2003a; Kumanogoh and Kikutani, 2003b). Sema4A-deficient mice develop normally but dendritic cells (DCs) and T cells from knockout mice display poor allostimulatory activities and T helper cell (Th) differentiation, respectively. As a result, *in vivo* antigen-specific T cell priming and antibody responses against T cell-dependent antigens are impaired in the mutant mice (Kumanogoh et al., 2005). Recently Yukawa and colleagues showed that Sema4A is in the nervous system as well. In primary hippocampal neurons, Sema4A induced growth cone collapse that could be blocked by Y-27632, a Rho-kinase inhibitor. Moreover, immunocytochemical analysis with antibodies against Sema4A demonstrated the binding of recombinant Sema4A to the growth cones of hippocampal neurons. This indicated that Sema4A could function as a chemorepulsive cue by activating a receptor whose signal is transmitted to Rho-kinase and induced growth cone collapse of hippocampal neurons (Yukawa et al., 2005). Semaphorins 4A, 4B, and 4C were expressed differentially in the primary olfactory pathway both during development and regeneration suggesting a role in directing olfactory receptor neurons (ORNs) from the epithelium and to the olfactory bulb, their target structure (Williams-Hogarth et al., 2000). Sema4B was also found to co-localize with PSD-95 at synaptic contacts between cultured hippocampal neurons (Burkhardt et al., 2005). In a similar manner, Sema4C and Sema4F also get recruited to synaptic terminals (Inagaki et al., 2001; Schultze et al., 2001). Developmental studies in Sema4A-deficient mice revealed abnormal morphology of photoreceptor outer segments during the time at which they establish contacts with apical microvilli of the retinal pigment epithelium (RPE) (Rice et al., 2004). The proline-rich region in the cytoplasmic domain of Sema4C associates

with SFAP75, a recently reported neurite outgrowth-related protein named Norbin. Western blot and immunohistochemical analyses with anti-Sema4C and anti-SFAP75 antibodies indicated that Sema4C and SFAP75 were enriched in the brain with a similar distribution pattern consistent with an interaction between Sema4C and SFAP75 (Ohoka et al., 2001). Sema4D has been shown to stimulate outgrowth of embryonic DRG sensory neurons *in vitro* (Masuda et al., 2004a). However, it is also known to act as an inhibitory factor for axonal regeneration when expressed in oligodendrocytes and myelin (Moreau-Fauvarque et al., 2003). Conrotto and colleagues have demonstrated that Sema4D is angiogenic *in vitro* and *in vivo* and that this effect is mediated by its high-affinity receptor, Plexin B1 (Conrotto et al., 2005). Sema4E was discovered to influence the outgrowth of branchiomotor axons in the pharyngeal arches in zebrafish (Xiao et al., 2003). Sema4F was to be expressed at high levels in the adult central nervous system and lung and to exhibit growth cone-collapse activity for retinal ganglion-cell axons. Moreover, it has been mapped to the motor neuron degeneration 2 disease loci (Encinas et al., 1999).

Sema5A and Sema5B lack the IgG domain that is found in semaphorins from subclasses 3 and 4 but instead have seven thrombospondin repeats followed by a short intracellular C-terminus which is unique for this subclass. Both semaphorins 5A and 5B were expressed, together with SEMA3A and 3C, in specific regions of early mouse embryos, suggesting a role in segregation of the developing somites or the undifferentiated neuroepithelium to distinct compartments. SEMA5A is present in axial and paraxial mesodermal tissues, limb bud, optic disc and nerve, whereas SEMA5B expression is restricted to the neuroepithelium along the entire antero-posterior axis (Adams et al., 1996). The transcription factor PAX6 which has been implicated in forebrain patterning, cerebral cortical area formation and in development of thalamocortical connections seems to regulate the expression of Sema3C and Sema5A (Jones et al., 2002). Additionally, Sema5A expression in cells of oligodendrocyte

lineage seems to contribute to the inhibition of CNS regeneration. Sema5A was expressed only by purified oligodendrocytes and their precursors, but not by astrocytes, and was present in both normal and axotomized optic nerve but not in peripheral nerves. Sema5A induced collapse of RGC growth cones and inhibited RGC axon growth when presented as a substrate *in vitro*. This has been confirmed by the use of function-blocking antibodies on optic nerve explants *in vitro* (Goldberg et al., 2004) and *in vivo* (Oster et al., 2003). Blocking of Sema5A function resulted in defasciculation of retinal axons causing them to leave the optic nerve, indicating that Sema5A normally helped ensheath the retinal pathway. SEMA5A maps to the gene deletion responsible for the Cri-du-Chat syndrome (Simmons et al., 1997; Simmons et al., 1998).

In 1998, the first semaphorin known to be associated with cell surfaces via a glycosylphosphatidylinositol linkage was identified. This was Sema K1 or Sema7A. It was found to be highly homologous to a viral semaphorin and could interact with specific immune cells, suggesting that like its viral counterpart it could play an important role in regulating immune function (Lange et al., 1998; Xu et al., 1998). In contrast to most previously described semaphorins, it is only weakly expressed during development but is present at high levels in postnatal and adult tissues, especially brain and spinal cord (Xu et al., 1998). SEMA7A is expressed in several structures of the rat embryonic brain and *in vitro* promotes the growth of numerous central axons originating from the vomeronasal epithelium, the olfactory epithelium, the olfactory bulb and the cortex as well as the dorsal root ganglia in the PNS. However, the disruption of SEMA7A gene in mice, lead only to minor defects in the lateral olfactory tract the axons of which failed to branch or project to the most caudal region of the olfactory cortex (Pasterkamp et al., 2003). Expression of Sema7A was demonstrated in lymphoid and myeloid cells, but no stimulation of cytokine production or proliferation was evident in B or T cells. In contrast, Sema7A is an extremely potent monocyte activator and is

less effective at stimulating neutrophils and coaxing monocytes toward dendritic cell morphology (Holmes et al., 2002). Additionally, Sema7A seems to be involved in the terminal innervation of the dentin-pulp complex too (Lallier, 2004; Maurin et al., 2005).

The Sema 6 class includes four membrane-bound members that lack the extracellular immunoglobulin domain (Gherardi et al., 2004; Kikuchi et al., 1997; Klostermann et al., 2000; Qu et al., 2002; Zhou et al., 1997). They display a relatively simple extracellular part, in which only the highly conserved Sema domain is present. Their intracellular tail is quite long compared to other semaphorin members, suggesting that this part in class 6 semaphorin might have unique and diverse functions. In mammals, four different class 6 family members have been identified (6A-6D) whereas, only three members have been identified in chick: Sema6A, 6B and 6D.

- Semaphorin 6A

Klostermann and colleagues reported the identification of the transmembrane semaphorin, Sema6A, in human and mouse (Klostermann et al., 2000). The human Sema6A gene encodes a protein of 1,030 amino acids with a calculated molecular mass of 112.2 kD. It consists of 20 exons covering approximately 60 kb of genomic sequence. The SEMA6A gene is localized to human chromosome 5q21-q22, which is known to be deleted in certain forms of lung cancer. Highest expression was observed in embryonic brain and kidney, whereas only low to moderate expression was seen in developing lung and liver. Only small amounts of Sema6A transcripts were detected in adult human tissues. Rather strong expression levels were detected in the placenta. Mouse neural embryonic tissues displayed high levels of Sema6A mRNA expression in proliferating zones, in the diencephalon, in the retina, in dorsal root ganglia, and in the trigeminal ganglion. Sema6A is expressed specifically in the developing cranial nerves, in the optic tract, in sensory axons, and in several tracts in the brain including the fasciculus retroflexus, stria medullaris, the anterior commissure, and thalamocortical axons

(Leighton et al., 2001). At early embryonic stages, expression is restricted to the ventral spinal cord, at later stages expression is also observed in the dorsal spinal cord in areas of lamina I and II. Sema6A is absent from all cervical and thoracic sympathetic ganglia. It is also expressed in skeletal muscles (Cohen et al., 2005; Zhou et al., 1997). Sema6A acts as a repellent on growth cones of sympathetic ganglia of E8 chicken embryos and NT-3 and NGF-sensitive DRG neurons *in vitro*, consistent with a traditional role as guidance signals (Xu et al., 2000; Zhou et al., 1997). However, the length of the cytoplasmic tail suggests that these semaphorins may also function as receptors. Via its zyxin-like C-terminal domain, Sema6A binds to the Enabled/Vasodilator-stimulator Phosphoprotein like proteins (EVL) that are involved in the cytoskeletal alterations associated with apoptosis and development. These findings suggested a role for transmembrane semaphorins such as Sema6A in retrograde signaling (Klostermann et al., 2000). Leighton and colleagues identified an *in vivo* guidance function for semaphorin 6A. An insertion was isolated in the SEMA6A gene (at amino acid 473 in the semaphorin domain) that completely abolishes wildtype Sema6A transcripts. Homozygous mutant mice were viable and fertile and displayed no obvious behavioral or morphologic phenotypes with the exception of aberrant development of the thalamocortical projections (Leighton et al., 2001). Thalamocortical axons projected normally in heterozygotes. Caudal thalamocortical axons projected abnormally down towards the amygdala region. Rostral projections appeared normal.

Elevated levels of SEMA6A mRNA in several renal tumor samples suggested a role in tumor neovascularization. The purified soluble extracellular domain (Sema-ECD) of human Sema6A blocked VEGF-mediated endothelial cell migration by inhibiting VEGF-mediated Src, Fak and Erk phosphorylation *in vitro.* Moreover, Matrigel assays showed that recombinant Sema-ECD inhibited both bFGF/VEGF and tumor cell-line induced neovascularization. Thus Sema6A seems to have therapeutic potential with respect to quenching growth factor

and tumor-induced angiogenesis (Dhanabal et al., 2005). Recently, Kerjan and colleagues have shown that Sema6A controls the initiation of radial granule cell migration in the mouse cerebellum. In SEMA6A mutant mice many granule cells remain ectopic in the molecular layer where they differentiate and are contacted by mossy fibers (Kerjan et al., 2005).

- Semaphorin 6B

The function of Sema6B is not very well understood. The expression appears quite early in development. The mRNA was first detected in the first branchial arch of embryonic day 11 (E11) rat embryos, and then subsequently in the myotomes and the dorsal root ganglia, from E11.5 through E13.5, but not in the brain. However, at E15, 18, 21 and P0, Sema6B was highly expressed in the brain, suggesting a role in both peripheral and central nervous system development (Kikuchi et al., 1997). In contrast to other semaphorins, Sema6B appears to be homogenously expressed throughout the entire spinal cord. In addition, Sema6B expression persists in adulthood in many tissues such as brain, heart, and lungs. Interestingly, Sema6B binds *in vitro* specifically to the SH3 domain of the proto-oncogene c-src suggesting that it can trigger intracellular signaling and act as a receptor (Eckhardt et al., 1997). Interestingly, the Sema6B transcript was downregulated in two different human glioblastoma cell lines (T98G and A172) upon prolonged treatment with all-trans-retinoic acid which is a known anti-tumor and differentiation-inducing agent (Correa et al., 2001). An affinity method developed by Collet and colleagues for the isolation of human genomic fragments containing binding sites for peroxisome proliferator-activated receptors (PPARs) and to identify novel PPAR target genes resulted in the identification of a sequence named ISF5148. This sequence was mapped to a position 8.5kb upstream of the human Sema6B gene. Moreover, expression of Sema6B in human glioblastoma T98G cells was strongly down regulated after treatment with clofibrate or Wy-14,643, two PPARα

agonists, suggesting the involvement of this gene in peroxisome proliferation and retinoic acid signaling pathways (Collet et al., 2004; Correa et al., 2001). Changes in Sema6B expression have been observed due to post-commissural fornix transection conducted at a time, when spontaneous axonal growth has ceased at the lesion site leading to the hypothesis that Sema6B may be a lesion-induced axonal growth inhibitor in the central nervous system (Kury et al., 2004).

- Semaphorin 6C

Kikuchi and his colleagues cloned Sema6C which was found to have a growth cone-collapsing activity on DRG neurons *in vitro* (Kikuchi et al., 1999). Sema6C is expressed in the rat spinal cord as well as in the dermamyotome, DRGs and the notochord during development. Later on, Sema6C mRNA is found also in cranial ganglia, the olfactory epithelium and the cerebellar plate. Postnataly, Sema6C expression is present in different cerebellar layers, pontine and inferior olive nuclei as well as in adult skeletal muscle tissue and many CNS structures. Thus, temporal expression of Sema6C in neurons and in their target areas during development suggests a potential role for this protein in axon guidance of motor and sensory neurons as well in directing commissural or cerebellar neurons. Due to alternative splicing, two isoforms of Sema6C were identified with tissue and age-dependent expression pattern (Kikuchi et al., 1999).

Human SEMA6C was cloned by Qu and colleagues in 2002 and was mapped to chromosome 1q12-21.1. In adult mouse tissues, Sema6C is expressed only in skeletal muscle, while in E13 embryos it was highly expressed in the areas of the lateral ventricle, the striatum, the wall of the midbrain, the pons/midbrain junction, and the choroid plexus. Three isoforms of Sema6C derived from alternative splicing were identified, and their expression was regulated in a tissue- and development-dependent manner. Deletion analysis indicated that the sema domain and the PSI domain are necessary for correct post-translation modification and subcellular localization. The extracellular

domain of Sema6C inhibited axonal extension of nerve growth factor-differentiated PC12 cells and induced the growth cone collapse of chicken dorsal root ganglion, rat hippocampal neurons, and rat cortical neurons in a dose-dependant manner(Qu et al., 2002).

- Semaphorin 6D

Sema6D, the last member of class 6 semaphorin to be characterized, exists in five different splice variants with tissue-specific expression patterns (Taniguchi and Shimizu, 2004). *In vitro* Sema6D has been shown to induce growth cone collapse of DRG and hippocampal neurons but had no effect on cortical neurons. It also inhibited axonal extension of NGF-differentiated PC12 cells. It has been mapped to chromosome 15q21.1 and is expressed abundantly in kidney, brain and placenta and moderately in the heart and skeletal muscles. It is expressed highly in the areas of the lateral ventricle, the striatum, the wall of the midbrain, the pons/midbrain junction, and the choroid plexus of E13 embryos (Qu et al., 2002). Sema6D in mice was detected on embryonic day 10.5 and its expression continued until birth. It was expressed predominantly in adult brain and lung, moderately in heart, small intestine, skeletal muscle, uterus, and placenta (Taniguchi and Shimizu, 2004). Sema6D knock down by siRNAs or overexpression in mice or chicken caused morphological abnormalities of the cardiac tube as well as of the neural tube, suggesting that Sema6D is involved in cardiac morphogenesis and in the formation of the neural tube (Toyofuku et al., 2004a; Toyofuku et al., 2004b). The observed phenotypes are in agreement with the expression pattern of Sema6D in normal mice where high levels of Sema6D are expressed in the developing heart and neural folds. This reveals a role for Sema6D in organogenesis apart from the effects on axonal growth.

iv. Receptors for Semaphorins

In general, the receptors for semaphorins are multimeric complexes composed of neuropilins and/or plexins (Chen et al., 1997; Eckhardt et al., 1997; Kolodkin et al., 1997; Pasterkamp et al., 1999; Pasterkamp and Verhaagen, 2001; Varela-Echavarria and Guthrie, 1997). Neuropilins and Plexins had been discovered many years earlier as antigens of monoclonal antibodies derived from the optic tectum of *Xenopus laevis* (Kameyama et al., 1996a; Kameyama et al., 1996b; Ohta et al., 1995; Ohta et al., 1992; Satoda et al., 1995; Takagi et al., 1991; Takagi et al., 1995). The fact that they are receptors for semaphorins was established only later. The membrane-bound semaphorins can bind directly to plexins, whereas secreted (class 3) semaphorins require neuropilins as co-receptors. In addition, other molecules have been identified that can transduce semaphorin signaling, including integrin receptors (Pasterkamp et al., 2003).

- Plexins

  The plexins are a large family of evolutionarily conserved transmembrane proteins, which are essential signal transducing components of most semaphorin receptor complexes (Tamagnone and Comoglio, 2000). All the plexins are large integral membrane proteins with a highly conserved cytoplasmic tail. Interestingly at their amino-terminus, they contain a highly conserved sema domain. In addition to the sema domain, the extracellular domain of Plexin is characterized by two or three Met-related sequence repeats (MRS). The large cytoplasmic moiety of Plexins contains a highly conserved plexin-specific Sex-Plexin (SP) domain, which is likely to trigger novel signal-transduction pathways and regulation of cytoskeletal structures by interacting with small GTPases (Conrotto et al., 2004; Conrotto et al., 2005; Raper, 2000; Rohm et al., 2000; Tamagnone and Comoglio, 2000). Initial insights into plexin function came from the finding that a viral semaphorin (A39R) utilized the B-lymphocyte protein VESPR (viral-encoded semaphorin receptor, now called Plexin C1) as a functional receptor (Comeau et al., 1998; Huber et al., 2003). Genetic

and biochemical evidence in *Drosophila* identified Plexin A as the functional receptor for the transmembrane semaphorin Sema1a, (Winberg et al., 1998). Subsequently nine different plexins have been identified in the mammalian genome, which can be sub-grouped into four different classes (A-D) (Tamagnone et al., 1999). Class A consists of four members (plexinA1-A4), while class B is composed of 3 members (plexinB1-B3). Only one member each has been identified for class C (plexinC1) and class D (plexinD1) (Fig 1.8).

The class-A plexins are the most extensively studied plexins (Fiore and Puschel, 2003; Murakami et al., 2001; Tamagnone and Comoglio, 2000). It has been found that the Sema domain at the N-terminus silences Plexin A1 signaling through intra-molecular interactions in the absence of ligands. This autoinhibition is released upon binding of Sema3A to the plexin/neuropilin complex (Takahashi and Strittmatter, 2001). Cumulative observations, including the fact that PlexinA3 mutant mice are deficient in class 3–secreted semaphorin repulsion (Cheng et al., 2001) establish that Plexins are components of the receptor complex for certain classes of Semaphorins. In mice, members of the PlexinA class are widely expressed in the central and peripheral nervous system and are spatio-temporally regulated.

PlexinA1 and PlexinA2 expression is limited to some neurons in the DRG (Murakami et al., 2001) whereas PlexinA3 seems to be expressed in all peripheral ganglia. In addition, PlexinA3 is expressed strongly in the whole spinal cord while PlexinA2 is expressed selectively in the dorsal spinal cord and PlexinA4 is the most abundant plexin in DRG (Suto et al., 2003). Targeted disruption of the PlexinA3 gene demonstrated its role in fasciculation of the ophthalmic branch of the trigeminal nerve. Furthermore, it regulates the development of hippocampal projections *in vivo* (Cheng et al., 2001). The vertebrate PlexinB1 and PlexinC1 have been shown to bind directly to Sema4D and the GPI-linked Sema7A, respectively (Takahashi et al., 1999; Tamagnone et al., 1999).

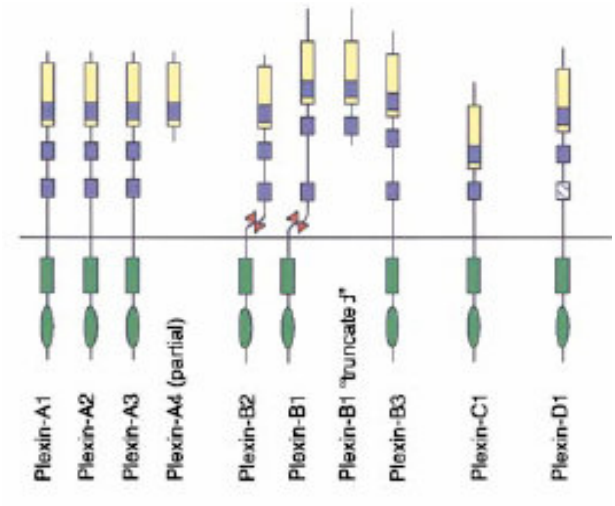


Fig: 1.8: Structural features of Plexins. In the extracellular moieties, yellow boxes indicate "*sema*" domains and blue boxes mark cysteine-rich MRS motifs, one of which is stippled to mark its atypical sequence. Potential furin-like proteolytic sites are marked by red ribbons. Plexin-B1 "truncated" is the product of a splicing variant. Plexin-D1 and plexin-C1 (VESPR) are more distant family members, since they include atypical features in their extracellular domains. The highly conserved intracellular domain of plexins (*SP domain*) contains two separate regions of high homology (green oval and box). Met-like receptors are disulfide-bound heterodimers and include a cytoplasmic tyrosine kinase domain (red box) (Tamagnone et al., 1999).

Sema4D/Plexin B1 signaling is required for Sema4D-mediated repulsion of hippocampal neurons *in vitro* (Swiercz et al., 2002). Apart from this very little is known about the role of PlexinBs *in vivo* (Giordano et al., 2002; Neufeld et al., 2005; Vikis et al., 2002; Vikis et al., 2000). However, PlexinB1 physically associated with Plexin A1, but not with PlexinA2 or A3, when their interactions were assessed using mammalian expression systems (Usui et al., 2003). PlexinD1 knock-out animals exhibit cardiac defects unrelated to cardiac crest migration. PlexinD1 may be essential in outflow tract septation, development of aortic arch artery and intersomitic vessels sprouting (Gitler et al., 2004). In addition to its role in heart development PlexinD1 has been implicated in the development of the vascular system consistent with its

predominant expression in endothelial cells (Gitler et al., 2004; Torres-Vazquez et al., 2004; van der Zwaag et al., 2002). Based on their expression pattern, it is clear that PlexinAs have functions that are independent of Neuropilins, because they are more widely expressed in the developing nervous system than Neuropilin-1 and -2 (Ohta et al., 1995) and Mauti et al., submitted). The function of plexins has been studied predominantly in context of their role as co-receptors (along with Neuropilins) for the secreted Semaphorins, class-3 Semaphorins (Fiore and Puschel, 2003; Huber et al., 2003; Takahashi et al., 1999; Tamagnone et al., 1999; Tamagnone and Comoglio, 2000).

- Neuropilins

  Neuropilins (Npn) were identified as receptors for Semaphorins concomitantly in two labs (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin and Ginty, 1997; Kolodkin et al., 1997). The neuropilins are transmembrane proteins with short, conserved, cytoplasmic tails that lack any known signaling motifs (Fig 1.9) and these are dispensable for propagating semaphorin repulsive signals (Nakamura et al., 1998). The extracellular domain of neuropilins contains two repeated complement-binding domains (CUB domains a1/a2 domains), two coagulation-factor homology domains (b1/b2 domains) and a juxtamembrane meprin/A5/mu-phosphatase (MAM) homology domain. While the CUB a1/a2 and b1/b2 domains seem to be essential to define the profile of semaphorin specificity, the MAM domain seems to be crucial for the functionally required neuropilin non-covalent oligomerization on the cell surface (Tamagnone and Comoglio, 2000). Two neuropilins genes have been identified in the genome of birds and mammals (Npn-1 and Npn-2); however, no neuropilin gene has been identified in invertebrates (He and Tessier-Lavigne, 1997; Kawakami et al., 1996; Kolodkin and Ginty, 1997; Kolodkin et al., 1997; Takagi et al., 1991; Takagi et al., 1995). The specific responses of different types of neurons to class 3 semaphorins can be explained by the restricted and unique neuropilin expression patterns, the preferential binding of individual class 3

semaphorins to Npn-1 and/or Npn-2, and also by the unique patterns of neuronal plexin expression observed during neural development (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin and Ginty, 1997; Murakami et al., 2001; Nakamura et al., 2000). The fact that neuropilins aggregate into dimers and the observation that Npn-1 forms heterodimers with Npn-2 when co-expressed, suggests a model whereby Npn-1 homodimers confer sensitivity to Sema3A; while Npn-2 homodimers are responsible for responding to Sema3F, heterodimerization may be required for responsiveness to Sema3C (Chen et al., 1997; Renzi et al., 1999; Takahashi et al., 1999). Comparative analyses of PlexinA1, PlexinA2 and PlexinA3 combined with neuropilin- 1 and neuropilin-2 showed that Sema3A and Sema3F signals are transduced effectively by PlexinA1 and A2 but not A3 (Takahashi and Strittmatter, 2001). In contrast, Cheng and colleagues showed that PlexinA3 mutant mice completely lose their responsiveness to Sema3F and that the responses of these neurons to Sema3A and of other neurons like hippocampal neurons to Sema3A and Sema3F are partially impaired. At present, it is unclear, how these findings can be reconciled. Still in summary plexins are required for neuronal responses to class 3 semaphorins together with neuropilins (Cheng et al., 2001).

Npn-1 is expressed in many classes of neurons, including most peripheral sensory neurons, autonomic neurons of the sympathetic ganglia, motor neurons in the spinal cord and the medulla, neurons in the hippocampal formation, retinal ganglion cells and olfactory neurons in the olfactory bulb (Kawakami et al., 1996). The expression of Npn-1 is developmentally regulated in both the peripheral and the central nervous systems. Npn-1 appears first in newly differentiated neurons and persists throughout the duration of active axonal growth fading only after the scaffolding of neuronal circuits has been established. Mice, carrying a null mutation for the npn-1 gene, are embryonic lethal and exhibit similar but stronger axon guidance defects compared to those observed in sema3A knockout mice.

Neuropilin-deficient mice show severe abnormalities in the trajectory of peripheral nerves. The neuropilin-deprived dorsal root ganglion neurons are perfectly protected from growth cone collapse elicited by Sema3A indicating that neuropilin-Sema3A-mediated chemorepulsive signals play a major role in guidance of PNS axons. Npn-1 mutant mice exhibit abnormal defasciculation of cranial nerves and peripheral nerves in the trunk, DRG cell clusters appear to be loosely arranged and sympathetic neurons are displaced.

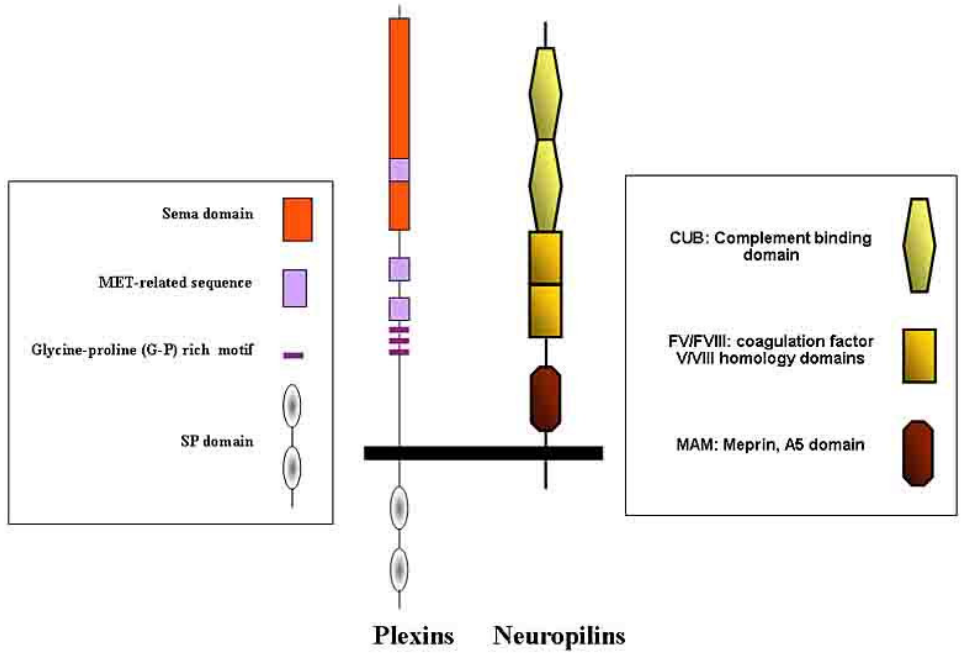


Fig: 1.9: Structure of Neuropilins and their Plexin co-receptors. The Nneuropilins and the Plexins act as receptors for the Semaphorin family members (Neufeld et al., 2005).

Interestingly, sympathetic neuronal precursors do not accumulate at their initial target sites around the dorsal aorta in npn-1 mutants (Fujisawa et al., 1997; Kitsukawa et al., 1997), a defect also observed in sema3A knockout mice (Kitsukawa et al., 1997; Taniguchi et al., 1997). A closer look at the DRGs revealed that most adult rat DRG neurons express Neuropilin-1 protein *in vitro* (Reza et al., 1999). However, the response of growth cones of these neurons (induced by recombinant Ccollapsin-1/Semaphorin3A and blocked by the anti-

Neuropilin-1 antibody) was restricted to those corresponding primarily to nociceptive sensory afferents. Neurotrophic factors had a differential effect on neuropilin-1 expression *in vitro*, with DRG neurons cultured in either NGF or GDNF expressing the highest levels on their neurites. These findings suggest that Neuropilin-1-mediated repellent effects of Semaphorins may regulate the behavior of nociceptive sensory axons in the adult as well as the embryonic peripheral nervous system (Reza et al., 1999). Investigation of the expression of Npn-1 mRNA in adult DRG neurons in intact and lesioned animals revealed a significantly increased level of Npn-1 mRNA expression only following sciatic nerve lesioning, but not after rhizotomy or dorsal funiculus lesioning. Furthermore, this upregulation was mainly confined to large diameter neurons of DRGs at lumbar levels L4/5, which provide the main sensory contribution to the sciatic nerve. These results suggest a role for Npn-1 in the axonal response to peripheral nerve injury, which may be specific to a particular subset of primary sensory neurons (Gavazzi et al., 2000). Neuropilin-1 and Sema3A knockout mouse embryos showed displacement of sympathetic neurons and their precursors and abnormal morphogenesis in the sympathetic trunk. Moreover, Sema3A suppressed the cell migration activity of sympathetic neurons from wild-type but not neuropilin-1 mutant embryos *in vitro* and instead promoted their accumulation into compact cell masses and fasciculation of their neurites. These findings suggest that the neuropilin-1-mediated Sema3A signals regulate the migratory pathways of sympathetic neuron progenitors and contribute to the formation of a stereotyped sympathetic nerve pattern (Kawasaki et al., 2002). Npn-1 deficiency is also associated with altered vascularization in the brain and a variety of defects in the large vessels of the heart outflow (Kawasaki et al., 1999), since Npn-1 also interacts with VEGF by increasing its affinity to its receptor. The strongest phenotype observed in Npn-1 mutants in comparison to Sema3A knockout mice might reflect the functional loss of more than one class 3 semaphorin and/or the loss of semaphorin-independent functions of Npn-1.

Npn-2 exists in six different isoforms that are generated by alternative splicing (Chen et al., 1997). Npn-2 is expressed in multiple areas in the developing CNS and PNS as well as many non-neural tissues. The expression pattern of Npn-2 partially overlaps with Npn-1 but is mostly complementary. In contrast to Npn-1, Npn-2 expression is not detected in the heart or in capillaries but is only found in the dorsal aorta. Unlike neuropilin-1, which binds with high affinity to Sema3A, Sema3C and Sema3F, neuropilin-2 shows high affinity binding only to Sema3C and Sema3F, not Sema3A (Chen et al., 1997; Giger et al., 1998). Npn-2 knockout mice are viable until adulthood and exhibit defects of axon fasciculation and targeting of selected cranial nerves and central projections (Chen et al., 2000; Giger et al., 2000). The mutants also lack the trochlear nerve and showed irregular trajectories of the occulomotor nerve but have no clear abnormalities in the projections and trajectories of spinal nerves. Recently it has been shown that expression of Npn-2 and PlexinA3 in cultured rat Schwann cells is diminished markedly by forskolin (an adenylate cyclase activator). Antibodies that recognize ectodomains of Npn-2 but not control antibodies prevented cultured Schwann cells from aligning in parallel and forming columns. These results are consistent with the view that in nerves undergoing Wallerian degeneration, Schwann cell-derived Npn-2 facilitates assembly of Schwann cells into the tubular aggregates (bands of Bungner) that guide regenerating axons (Ara et al., 2005).

- Semaphorin signaling

  A broad summary of the semaphorin signaling modes is described in Fig 1.10. Membrane-bound vertebrate Semaphorins bind directly to Plexins, whereas secreted Semaphorins (class 3) also require Neuropilins as obligate co-receptors. Several lines of evidence indicate that the cytoplasmic domain of Plexins is required for Ssemaphorin signaling, whereas the small cytosolic tail of neuropilins is dispensable.

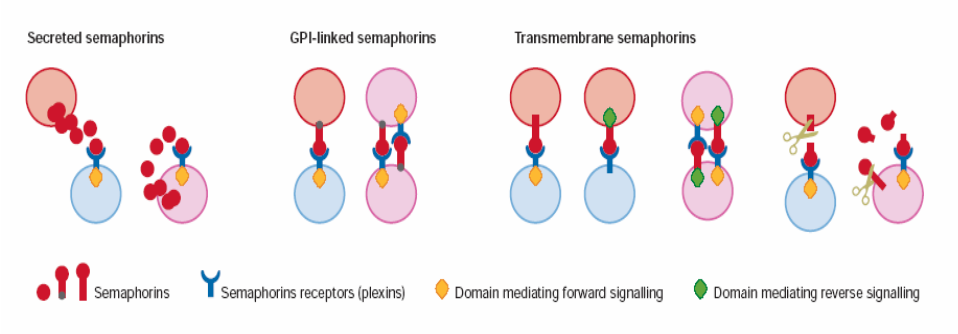


Fig: 1.10: Semaphorin signaling modes. Semaphorins and their receptors might be expressed on distinct cell populations (shown in red and blue, respectively) or be co-expressed (pink). Secreted semaphorins mediate either paracrine or autocrine signals. Apart from classical forward signals, transmembrane semaphorins could also trigger reverse signaling (mediated by their cytoplasmic domain) or be released into the extracellular space by proteolytic cleavage and so behave as secreted ligands (Tamagnone and Comoglio, 2004).

A recent study, however, revealed an independent functional role for the cytoplasmic tail of Npn-1, which is probably mediated through its PDZ (for PSD95, Discs-large and ZO1)-domain binding sequence (Wang et al., 2003). Neuropilin-1 has been shown to bind to a protein called NIP (Neuropilin-1 Interacting Protein). It is involved in regulating specificity of neuropilins by altering the conformation of the protein (Cai and Reed, 1999). CD72 and Tim2 were found to interact functionally with transmembrane semaphorins in the immune system. They are not related to the family of plexins and neuropilins (Kumanogoh et al., 2002; Kumanogoh et al., 2000). GPI-linked Sema7A is known to bind to Plexin-C1 (Tamagnone et al., 1999) but it also has Plexin-independent activity that is mediated by integrin-β1(Pasterkamp et al., 2003). Most of the interactions between Semaphorins, Plexins, Neuropilins and other proteins have been extensively reviewed by Tamagnone and Comoglio in EMBO Reports, 2004 (Fig 1.11). Recent studies have shown that VEGF can act as a survival and chemotactic factor for cancer cells in a VEGF-receptor-independent manner, probably by antagonizing the activity of Semaphorins that is mediated by Neuropilin/Plexin complexes (Bachelder et al., 2003). GTPases of the Rho family are candidate

signal transducers of the plexins; however, evidence of the direct mechanisms through which they are involved is lacking. Recent findings indicate that plexin signaling regulates integrin-based adhesion, although the molecular mechanisms still need to be defined (Barberis et al., 2004; Oinuma et al., 2003; Swiercz et al., 2002; Swiercz et al., 2004).

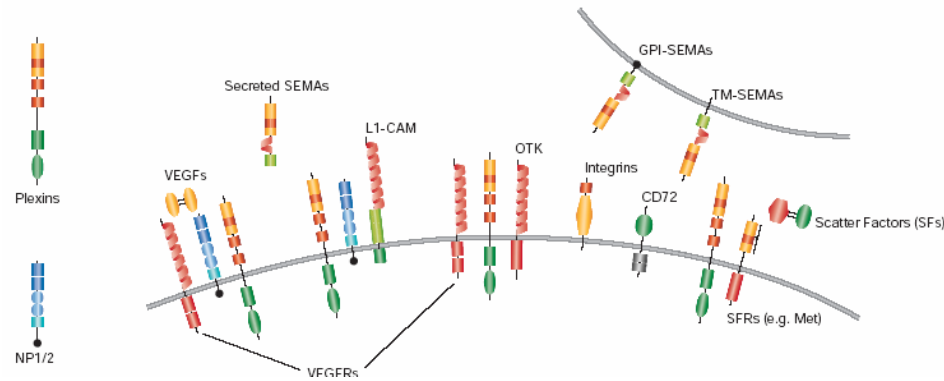


Fig: 1.11: Semaphorin receptor complexes. Plexins bind Semaphorins (SEMAs) and can form receptor complexes with Neuropilin 1 and 2 (NP1/2), with cell adhesion molecule L1 (L1-CAM) and with receptor tyrosine kinases, such as Off-track kinase (OTK), Scatter-factor receptors (SFRs) and Vascular endothelial growth factor receptors (VEGFRs). Neuropilins act as co-receptors for both secreted Semaphorins and VEGFs. Integrins are receptors of extracellular matrix components, but Integrin-β1 also mediates Sema7A activity. CD72 is a low affinity receptor for Sema4D.GPI, glycosylphosphatidylinositol; TM, transmembrane (Tamagnone and Comoglio, 2004).

1.2 Assembly of neuronal circuitry in the spinal cord

The exclusive capability of distinct classes of neurons to assemble into defined neuronal circuits reflects the function of molecular properties that these neurons acquire from the earliest stages on and during their entire differentiation process. Molecular features that distinguish different classes of neurons coordinate cell body migration, direct axonal projections towards the target region and control the precise formation of synaptic connections. The molecular specification of individual subsets of neurons occurs sequentially, involving the

progressive restriction in the developmental potential of progenitors as well as postmitotic neurons (Edlund and Jessell, 1999).

- Motor neurons
  - Motor neurons arise from initially uncommitted, dividing active ventral progenitors in the ventricular layer of the neural tube. The molecular basis of motor neuron differentiation has been most extensively investigated in chick and mouse embryos (Jacob et al., 2001; Jessell, 2000; Liu et al., 2001). Commitment to motor neuron identity involves a progressive restriction of progenitor fate imposed by a ventral-to-dorsal gradient of an extrinsic signal, Sonic hedgehog (SHH), which is produced by the adjacent notochord and the floorplate. Graded sonic hedgehog signaling establishes multiple progenitor domains within the ventral neural tube. Each domain in turn gives rise to distinct neuronal classes, by activating or repressing the expression of a key set of transcription factors at different concentration thresholds. Once the motor neuron progenitor domain is established, committed precursors initiate an autonomous, homeodomain protein-mediated program of differentiation, marked by independence from sonic hedgehog signaling (Jessell, 2000). After motor neurons have left the cell cycle, they acquire columnar subtype identities that have classically been revealed by the position of motor neuron cell bodies in the spinal cord and by the pattern of motor axon projections in the periphery (Landmesser, 1978a; Landmesser, 1978b; Tosney et al., 1995). Five major columnar groups of motor neuron can be recognized on the basis of these criteria. Two of these groups are found within the medial motor column (MMC): a set of medial MMC (MMC_M) neurons that is generated at all rostrocaudal levels of the spinal cord and that extends axons to axial muscles. At thoracic levels, a set of lateral MMC neurons is generated that project their axons to body wall muscles (Tosney et al., 1995). A third set, pre-ganglionic autonomic motor neurons [termed Column of Terni (CT) neurons in chick], is also generated selectively at thoracic levels and these neurons project axons to sympathetic targets (Prasad and Hollyday, 1991). The final

two columnar groups are found within the lateral motor column (LMC) at limb levels of the spinal cord: medial LMC (LMC_M) neurons project axons to ventral limb muscles and lateral LMC neurons project their axons to dorsal limb muscles (Landmesser, 1978b; Tosney et al., 1995). In the chick, the molecular pathway of spinal motor neuron differentiation is triggered by auto-activation of MNR2, acting as a selector gene, in motor neuron precursors during their terminal mitotic cycle. Misexpression of MNR2 in the dorsal spinal cord is sufficient to induce ectopic somatic motor neuron differentiation (Tanabe et al., 1998). Fiver LIM domain proteins, namely Islet-1, Islet-2, Lim1 and Lim3, Lhx3, are expressed combinatorially by distinct motor neuron subtypes, which are distinguished by their peripheral axon trajectories and settling position within medial or lateral subdivisions of two major longitudinal columns in the ventral spinal cord (Tsuchida et al., 1994). A series of misexpression studies in the chick showed that MNR2 induces Islet-1 expression and cooperates with the latter protein in activating Islet-2; MNR2 also lies upstream of Lhx3 which in turn activates HB9 coordinately with Islet-1 (Tanabe et al., 1998). Islet-1 is expressed by all motor neurons (Ericson et al., 1992). In mutant mice lacking Islet-1 function, there is a block in the differentiation of all classes of motor neurons which die as soon as they become post-mitotic (Pfaff et al., 1996). Therefore, Islet-1 activation represents an early critical point of convergence in the cascade of molecular events that promote motor neuron diversification and survival. Misexpression of Islet-1 in the spinal cord does not lead to the ectopic generation of motor neurons, suggesting that Islet-1 is necessary but not sufficient to instruct motor neuron differentiation (Tanabe et al., 1998). Lhx3 and the highly related gene, Lhx4 in co-ordination, determine two important phenotypic characteristics, namely cell body migration pattern and ventral axon trajectory. Combined inactivation of Lhx3 and Lhx4 results in the acquisition of an inappropriate identity by LMC, MMC and spinal visceral motor (VM) neurons, reflected in dorsal cell body migration and dorsal axon trajectories, which are normally displayed only by branchiomotor /visceral motor (BM/VM) motor neurons of the hindbrain

and a distinct subpopulation of motor neurons in the rostral spinal cord (Sharma et al., 1998). In keeping with a key role in determining columnar identity, the singular activity of Lhx3 is sufficient to instruct binary choices between all other spinal motor neuron identities. Forced maintenance of Lhx3 expression in spinal VM, LMC and lateral MMC (MMC_L) motor neurons endows them with an MMC_M-like identity, with concomitant rerouting of motor axons to axial muscles. By contrast, Lim-1, which is activated in post-mitotic lateral neurons of the LMCs (LMC_L), is not necessary for the specification of LMC_L identity and its function is confined to regulating axon pathfinding decisions in the periphery (Kania et al., 2000). Finally, Islet-2 is expressed by MMC_L and LMC neurons, but its role in motor neuron differentiation has yet to be elucidated by loss- and gain-of function studies. In summary, the differential activation of homeodomain transcription factors is causally linked to the progressive refinement of motor neuron identity. Thus, disruption of the transcriptional program of differentiation early in development affects motor neuron specification or columnar identity, whereas later disruption affects more restricted features of motor neuron phenotype. The presence of multiple motor neuron subtypes at the same axial level that are derived from progenitors with identical dorsoventral locations raises the question of how this diversity is generated. LMC_L neurons migrate past early-born LMC_M neurons which show retinoid activity. Retinoic acid (RA) induces differentiation of LMC_L neurons *in vitro*, down-regulating Islet-1 and up-regulating Lim-1 that distinguishes LMC_L from LMC_M neurons (Sockanathan and Jessell, 1998). The topographic and functional organization of spinal motor neurons is established during successive phases of specification and differentiation and correlates with selective patterns of expression of various families of transcription factors (Lee and Pfaff, 2001). The pool identity of individual motor neurons can be defined on the molecular level in part by the status of expression of ETS domain transcription factors, notably Er81 and Pea3 (Lin et al., 1998; Sharrocks, 2001). The initiation of expression of these two ETS proteins within different motor neuron pools appears to be tightly

regulated by the availability of peripheral signals (Lin et al., 1998). Local signaling interactions between post-mitotic motor neurons therefore represent an additional modality in the acquisition of motor neuron subtype identity. At a finer level of resolution, further distinctions in motor neuron subtype identity are evident. Within individual columns, motor neurons innervating a single muscle are organized into discrete pools consisting of some hundreds of neurons (Landmesser, 1978b) which can be defined by their combinatorial expression of LIM domain proteins and members of the ETS (Lin et al., 1998) and forkhead (Dou et al., 1997) classes of transcription factors.

- Dorsal root Ganglia (DRG) neurons

  - A subset of multipotent neural crest cells migrates along stereotypic pathways and coalesces at specific locations to form the spinal sensory ganglia also called dorsal root ganglia (DRG). Spinal sensory neurons comprise a morphologically and functionally heterogeneous group of neurons, specialized in the transfer of different sensory signals (Farinas et al., 2002). During development primary sensory neurons originate from progenitors that migrate from the neural crest and certain ectodermal placodes to the sites where sensory ganglia form (D'Amico-Martel and Noden, 1983; Lindsay and Rohrer, 1985). The axonal projection of DRG neurons is a useful model to study axon guidance mechanisms. During the initial stage of DRG axonal growth, surrounding “non-target” tissues such as the dermamyotome, the notochord, and the ventral spinal cord release strong chemorepulsive signals for DRG axons (Keynes et al., 1997; Nakamoto and Shiga, 1998). Two axonal processes grow in opposite directions from the cell bodies of these early neurons to reach their peripheral and central target fields. As in other parts of the nervous system, the innervation of these target fields is associated with a period of neuronal death during which the superfluous neurons are eliminated, followed by a period of modification and refinement of connections. Each DRG innervates a full array of targets in the periphery, including skin, muscle, and viscera. Individual DRG neurons connect to specific

types of sensory receptors, conveying information about position in space (proprioception), pain (nociception), distension, or touch (mechanoception) to the CNS. Neurotrophins play an essential role in the maintenance of a normal complement of neurons since all sensory neurons require the presence of at least one neurotrophin during development. Although the neurotrophic hypothesis postulates that neurons become dependent on a particular neurotrophin when their axons encounter their final targets there is evidence demonstrating that neurotrophins are expressed during early development before axon-target recognition and are therefore also implicated in gangliogenesis (Buchman and Davies, 1993; Farinas et al., 2002). Sensory fibers innervating the hindlimb are established in a precise orderly manner (Honig, 1982). During normal development, sensory axons appear to grow on the motor axons after they exit the spinal cord and always project to the same muscles as the neighboring motor neurons (Tosney and Landmesser, 1985b). Manipulations of neural tube, including the neural crest, or of the hindlimb before axonal outgrowth showed that sensory neurons projected incorrectly, if their corresponding motoneurons made wrong connections in the extremities (Honig et al., 1986; Landmesser and Honig, 1986). Incidentally, sensory neurons innervating skin or muscle in the periphery appear less rigidly specified than motoneurons and have more flexibility in their pathway and target choices. At the stages when innervations are being established, cutaneous as well as muscle afferents, unlike motoneurons, may not yet have acquired specified identities and the ability to recognize and respond selectively to their appropriate targets (Adams and Scott, 1998). Notochord-derived repellants act in a spatially and temporally specific manner to shape the initial trajectories of DRG axons (Masuda et al., 2004b). Central projections of sensory neurons follow a strict spatio-temporal pattern with different DRG neurons having central arborizations in the spinal cord that are specific for the sensory modality. In chicken, cutaneous and muscle axons of sensory afferents reach the spinal cord by stage 23, stalling there for 24 hours in the primordium of the dorsal funiculus before extending axons rostrally as

well as caudally. At around stage 28, central projections begin to enter the gray matter of the spinal cord. While cutaneous afferents branch frequently remaining in the dorsal horn (Mendelson et al., 1992), proprioceptive axons reach the vicinity of motor neuron dendrites without branching and form functional contacts around stage 32 (Davis et al., 1989). Netrin-1 and chick UNC5 homolog-3 are differentially expressed by early cutaneous and proprioceptive neurons, while neogenin is expressed in all DRG neurons (Guan and Condic, 2003). The sensory afferents that supply muscle spindles (called Ia afferents) project virtually unbranched through the medial half of the spinal cord and arborize in the ventral cord where they make direct synaptic contacts with the motor neurons (Ozaki and Snider, 1997). Both types of sensory neuron projections (peripheral and central) are established precisely and correctly from the outset, and neither cell death nor retraction of axons plays a role in the development of appropriate connectivity. The segregation of afferent inputs into laminar-specific projections is dependent on diffusible factors, integral proteins and/or extra cellular matrix proteins (Ozaki and Snider, 1997; Sharma and Frank, 1998).

- Commissural Neurons
  - In a wide variety of bilaterally symmetric organisms, sensory information is transferred from one side of the body to the other through axon commissures formed by interneurons that extend axons across the ventral midline (Fig 1.12) (Eide et al., 1999; Tear, 1999). In vertebrates, commissural neurons located within a dorsal region of the developing spinal cord initially project axons along a stereotypic pathway toward the ventral midline (Bovolenta and Dodd, 1990; Colamarino and Tessier-Lavigne, 1995b). The roof plate and the floorplate, specialized structures that are situated at the dorsal and ventral midline, respectively, appear to play reciprocal roles in the ventral migration of commissural axons/growth cones (Kaprielian et al., 2001).

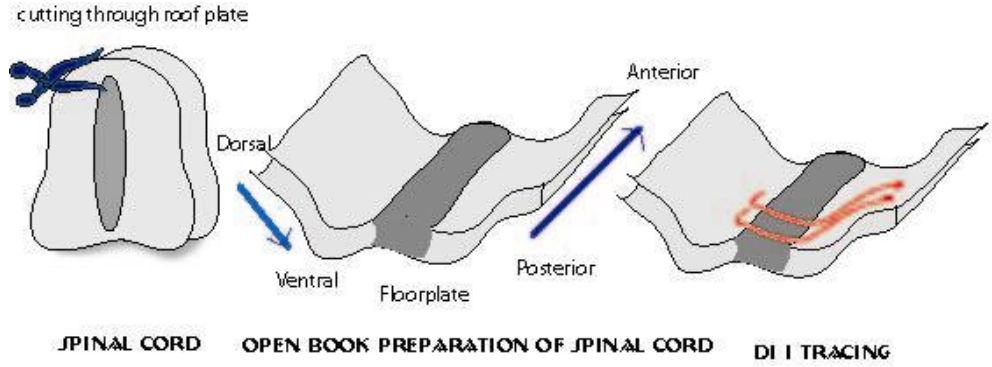


Fig: 1.12: Commissural neuron trajectory. Commissural neurons located in the dorsolateral part of the spinal cord extend their axons ventromedially toward the floorplate. Once they reach the floorplate, they cross the ventral midline and turn rostrally along the longitudinal axis.

Netrin 1, a soluble chemoattractant secreted by floorplate cells (and by cells situated in the ventral ventricular zone) and presumably distributed along a dorsoventral (DV) gradient, guides DCC-expressing commissural axons ventrally (Fazeli et al., 1997; Keino-Masu et al., 1996; Leonardo et al., 1997; Placzek et al., 1990; Serafini et al., 1996). In complementary fashion, the ability of bone morphogenetic protein (BMP) 7, a TGFβ super-family member secreted by cells comprising the roofplate, to repel commissural axons *in vitro* suggests a possible role for this structure in orienting these axons away from dorsal regions of the spinal cord during the first phase of axon outgrowth (Augsburger et al., 1999). Upon reaching the ventral midline, commissural axons cross over to the contralateral side of the spinal cord by navigating through the floorplate. After exiting this structure, these axons make an orthogonal turn and join other types of axons extending within the ventral funiculus (Colamarino and Tessier-Lavigne, 1995b; Kaprielian et al., 2001). A direct interaction between Axonin-1 (the avian ortholog of rodent TAG1) expressed by commissural axons and NrCAM expressed by floorplate cells was found to regulate the entry of commissural axons into the floorplate (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997), through a mechanism that apparently occurs independently of axon elongation (Fitzli et al., 2000). Perturbation experiments with F-spondin, an extracellular-matrix molecule secreted

by floorplate cells, revealed a requirement for F-spondin to prevent the lateral drifting of the commissural axons after having crossed the floorplate (Burstyn-Cohen et al., 1999). More recent studies have demonstrated that commissural axons exhibit a variety of midline pathfinding defects, including stalling within the floorplate and rostrocaudal polarity errors at the contralateral floorplate margin in mice deficient in neuropilin-2 (Zou et al., 2000). Several studies performed in Drosophila, chicken embryos and mice have already identified a great number of both diffusible and contact-dependent guidance cues that can guide commissural axons towards the floorplate and mediate midline crossing (Stoeckli, 1998). Lyuksyutova and colleagues tested the effects of different molecules on the behavior of commissural growth cones in spinal cord explants. Of those tested, several members of the Wnt family of secreted signaling molecules were able to affect the growth of commissural axons after they crossed the floorplate. Of the several Wnt family members expressed in the spinal cord, Wnt4 had an increasing posterior-to-anterior gradient of RNA expression. Thus, Wnt4 was concluded to attract postcommissural axons rostrally (Lyuksyutova et al., 2003). In our lab, a new role for the morphogen Sonic hedgehog (Shh) has recently been found in postcommissural axon pathfinding. Similarly to Wnt4, Shh acts as an instructive guidance cue for postcommissural axons and directs them along the longitudinal axis (Bourikas et al., 2005). However, in contrast to Wnt4, Shh acts as a repellant.

1.3 Hindlimb innervation

Motor axon pathfinding occurs in a stepwise manner and is dependent on the differential action of guidance cues, which are serially recruited at discrete locations along the axonal pathway. It is divided into following stages: axonal exit from the CNS, growth along a shared common pathway and navigation to and away from different choice points (Schneider and Granato, 2003). The first step in a motor axon's pathway is to correctly exit the CNS and project its axon into one of the

segmental nerves, connecting the CNS to the periphery. Motor axons grow initially away from the floorplate and penetrate the neuroepithelium at specific exit points. Co-culture experiments *in vitro* demonstrate that all classes of motor axons are repelled when placed adjacent to floorplate cells (Guthrie and Pini, 1995). Motor axons form a single ventral root within each somite to leave the spinal cord. The somites are segmented blocks of mesoderm which become partitioned into sclerotome and dermamyotome components. Motor axons emerging from the spinal cord at exit points traverse the sclerotome component of the somite only within its rostral half. Repulsive and attractive activities, derived from the caudal and rostral halves of the sclerotome respectively, impose the periodic arrangement of motor nerves exiting from the spinal cord (Keynes and Stern, 1984). The process of axon initiation is relatively rapid and synchronous and by stage 23-24, all eight spinal nerves have reached the base of limb and are beginning to form the crural and sciatic plexuses characteristic of the hindlimb (Fouvet, 1973; Lance-Jones and Landmesser, 1980; Tosney and Landmesser, 1985a). Axons invade the limb bud and begin to form functional connections at stage 27 (Landmesser, 1978b; Landmesser and Morris, 1975) prior to muscle cleavage. At this time motoneurons become dependent on their peripheral target (Hamburger, 1958; Hamburger, 1975; Hamburger, 1977) and during a period of naturally occurring cell death (stage 29-35), their number is reduced by more than half (Chu-Wang and Oppenheim, 1978; Hamburger, 1975). Different classes of motor neurons innervate different muscle targets following predefined pathways. For example, motor neurons of the MMC_L send axons to the epaxial muscle, whereas other motor axons of the MMC_M avoid the dermamyotome and navigate ventrolaterally to innervate hypaxial muscles (Shirasaki and Pfaff, 2002). Axons that arise from each motoneuron pool exit the spinal cord by the adjacent spinal nerve and follow stereotyped anatomical pathways (Fig 1.13). Axons within spinal nerves 1-3 and occasionally thoracic (T) 7 converge to form an anterior or crural plexus. Dorsal and ventral nerve

trunks and muscle nerves emerging from this plexus project to anterior thigh regions. Some axons within LS spinal nerve 3 and axons within LS4-8 converge to form a posterior or sciatic plexus. Nerve trunks and muscle nerves emerging from the sciatic plexus project to posterior thigh regions and to more distal limb regions.

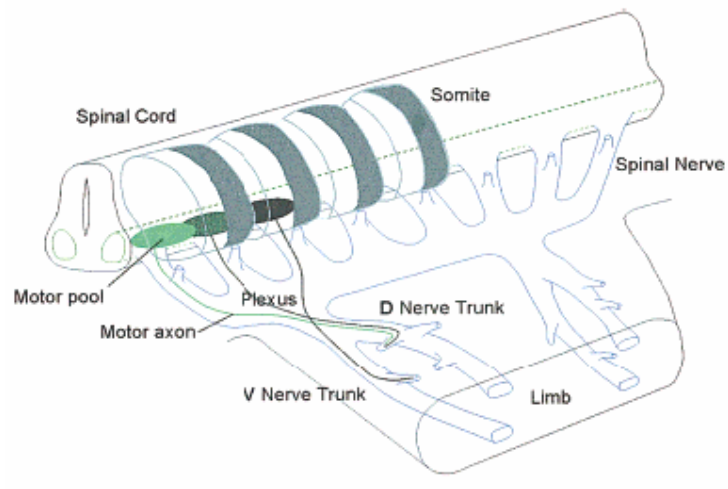


Fig: 1.13: Motoneuron projections into the embryonic chicken hindlimb. Motor axons originating from different motor pools within the cord grow through the rostral half, and avoid the caudal (shaded grey) part of the somite, to form the spinal nerves. Sorting occurs within the plexus region whereupon motor axons segregate into either the dorsal (D) or ventral (V) nerve trunks as they enter the limb (Jacob et al., 2001).

Within these trajectories, axons originating from different pools may converge and cross one another in contributing to a dorsal and ventral nerve trunk and finally to individual muscle nerves (Lance-Jones, 1988; Lance-Jones and Landmesser, 1981; Tosney and Landmesser, 1985b). Motor axons in each of these nerve trunks grow to the base of the limb, called the plexus area, where they pause for 24 hours before entering the limb bud. Apparently, axons wait in the plexus for limb maturation to occur (Varela-Echavarria et al., 1997; Wang and Scott, 2000). Within the plexus region, axon trajectories are highly individualistic with many abrupt turns, perhaps reflecting a process of active sorting (Tosney and Landmesser, 1985a). This step seems to be dependent on target-derived chemoattractants such as HGF and guidance cues produced in

the developing limb. Additionally, some tissues act as barriers to axons as they navigate to the hindlimb. Motor axons seem to avoid the perinotochordal mesenchyme and the pelvic girdle precursor tissue (Oakley and Tosney, 1991; Tanaka, 1991; Tosney and Oakley, 1990). Once motor axons are near their target muscle, they have to recognize and form synapses with the appropriate muscle fiber (Laskowski and Sanes, 1987; Laskowski and Sanes, 1988). The different molecules involved in motor axon pathfinding cues, such as Semaphorins, Neuropilins, Plexins, Slits, Robos, members of the Eph family, extracellular matrix molecules, Hepatocyte Growth Factor/Scatter Factor, peanut agglutinin-binding glycoproteins, neural cell adhesion molecules, and various other molecules that are responsible for the precise connectivity of the peripheral nerves in the hindlimb have been reviewed by (Krull and Koblar, 2000).

1.4 Defining the CNS/PNS interface

The demarcation of boundaries between different functional compartments is an important feature of neural development. A critical property of these compartments is the maintenance of segregated cell populations in their boundaries during development. However, the same boundaries should also be permeable to growing axons that make functionally significant connections between distinct compartments. The molecular and cellular basis underlying the establishment of these boundaries between the central and the peripheral nervous system are not very well understood.

- Border controls at CNS/PNS interfaces
  - CNS and PNS compartments meet at specialized transition zones. These are located both at the dorsal root entry zone (DREZ), where the afferents of primary sensory dorsal root ganglion (DRG) neurons enter the spinal cord via the dorsal roots and at the ventral motor exit point (VMEP), where motor axons leave the cord via the

ventral roots. The mature CNS/PNS interface at these sites is characterized by the cellular apposition of astrocytes and Schwann cells, glial cells derived from the neural tube and neural crest, respectively. During development, when barriers between the central and peripheral nervous systems are less rigid than in the adult stages, it is vital that each retains its integrity. The formation of a tight barrier between the CNS and the periphery is a feature common to all vertebrate nervous systems. In the uninjured adult, the blood–brain barrier prevents diffusion of most large molecules and the migration of cells such as circulating peripheral lymphocytes or CNS cells themselves. During development, the situation is less rigid. Many neurons of the PNS originate from the dorsal neural tube. During a late phase of neurulation, waves of migratory neural crest cells are generated at the dorsal midline of the developing neural tube. In addition, axons carrying efferent signals and afferent signals cross the boundary between CNS and PNS in large numbers. At spinal cord levels, they do this through the VMEP and DREZ, respectively. There is no absolute barrier for contacts or interactions between central and peripheral cells, and in fact no continuous basal lamina to separate the two compartments. Nevertheless, there is no mixing of cell bodies between the two (Fraher, 1997).

Every nerve that leaves the CNS has a transition zone (TZ) (Fig. 1.14). In vertebrates, most TZs examined have a glial partition stretching across the nerve bundle. This forms the CNS/PNS boundary and is penetrated by the axons as they cross between the CNS and PNS. There is a sharp discontinuity of tissue types at the TZ. The myelinating glia meets at the transitional node, where features of central and peripheral nodes are combined. The main supporting cells are astrocytes centrally and endoneurium peripherally. The interface between PNS and CNS lies at the surface of the CNS and consists of the superficial plasmalemmae formed by astrocyte processes which form the surface of the glia limitans. This is covered by a basal lamina, which in turn is continuous with each of the sheaths forming the inner

elements of the endoneurial tubes around the nerve fibers in the PNS. At the TZ, the CNS tissue extends distally into most roots as a tapering central tissue projection. The astrocytic covering of the TZ is a thickening of the glia limitans generally. Axons penetrating the glial barrier formed by the TZ glia limitans, do so at a very early stage of development (Fig 1.14a) (O'Brien et al., 2001; O'Brien et al., 1998). The axons of the bundles themselves are naked and are apposed to one another at first. They are secondarily segregated by fine astrocytic processes which grow in from outside the margins of the bundle (Fig 1.14b&c). These become progressively more elaborate and form the thick, highly complex mosaic which characterizes the TZ glia limitans. As a result of the segregation process, myelinated axons cross the TZ barrier singly. The point at which they pierce the glia limitans coincides with the locus of the transitional node. Most non-myelinated axons cross the TZ in a different way (Fraher, 2002). These transition zones at the DREZ and the VMEP can be considered as the controllers between the CNS and the PNS and the investigation of the molecular composition of this area would shed light on how this interface is built and maintained.

In development the earliest manifestation of these interfaces are neural crest derivatives called boundary cap (BC) cells that cluster at the future VMEP and DREZ (Fig 1.15).

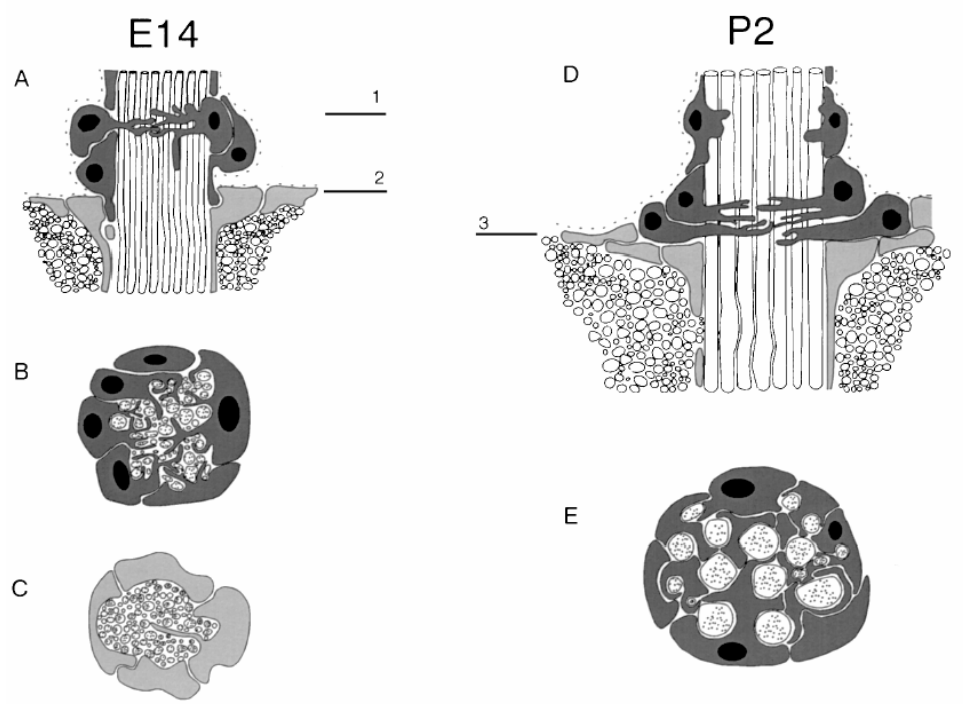


Fig: 1.14: Proposed mechanism for the formation of a perforated glial barrier at the Transition Zone (TZ). (A) Schematic view of a longitudinal section through an axon bundle at E14 passing through the cord surface. At the ventral rootlet level, branching processes extend between the axons from clustered cells at the bundle surface to form an interlocking matrix segregating them. (B) Cluster cells and matrix shown in a transverse section at level 1 in (A). (C) Transverse section of the bundle at the cord surface (level 2 in A) with little segregation of the axons. (D) Shortly after the end of gestation, e.g. at P2, associated with radial growth of the cord, the cell clusters have become displaced. They now lie at the cord surface and surround the TZ. (E) At this stage, shown in a transverse section at the level of the cord surface level 3 in D, processes form the matrix which completely segregates the axons at the TZ (Fraher, 1997).

- Boundary cap cells

  The neural crest gives rise to most of the components of the PNS, including the glial satellite and the Schwann cells. In addition, neural crest cells also give rise to another type of PNS glial cells, the boundary cap (BC) cells (Niederlander and Lumsden, 1996). These

latter cells are located at the interface between CNS and PNS, at the exit/entry points of ventral motor and dorsal sensory axons. Little was known about the formation and the role of this interface between PNS Schwann cells and CNS astrocytes. In order to understand the function of BC cells different transgenic mouse lines were designed, taking advantage of the fact that Krox20 was a well-known marker for BC cells in the mouse (Schneider-Maunoury et al., 1993; Topilko et al., 1994; Wilkinson et al., 1989). Two knock-ins into the Krox20 locus were obtained. In the first one, the Cre recombinase gene was inserted in place of Krox20 which allowed the specific activation or elimination of genes specifically in Krox20-expressing BC cells (Taillebourg et al., 2002; Voiculescu et al., 2000; Voiculescu et al., 2001). In the second line, Krox20 was replaced by a cassette containing the green fluorescent protein (GFP) gene, flanked by lox P sites and followed by the gene of the A chain of the diphtheria toxin. In this latter case, the GFP is normally expressed in Krox20-expressing cells, allowing their easy identification and purification. Upon expression of the Cre recombinase, the GFP gene is excised and the gene for the A chain of the diphtheria toxin is activated, resulting in the elimination of Krox20-expressing cells (Vermeren et al., 2003). This allowed for specific ablations of BC cells *in vivo*. Targeted ablation of BC cells has provided evidence that they are responsible for preventing inappropriate cell mixing between emerging CNS and PNS compartments at the VMEP. Hence, in the absence of BC cells, spinal motor neurons escape the spinal cord by translocating along their axons into ventral roots (Vermeren et al., 2003).

•

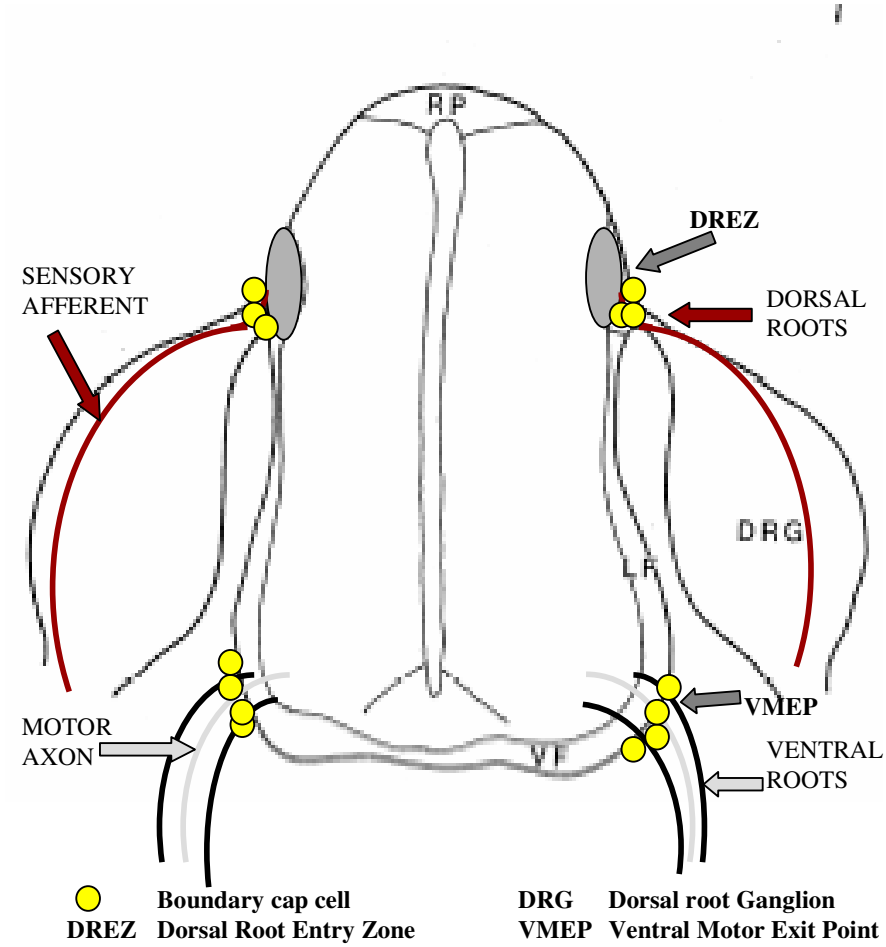


Fig: 1.15: Schematic representation of boundary cap cells located at the dorsal root entry zone (DREZ) and at the ventral motor exit point (VMEP). Sensory afferents enter the dorsal part of the spinal cord at the DREZ and the motor axons exit the spinal cord at the VMEP. Boundary cap (BC) cells (shown in yellow) are clustered at these entry and exit sites.

The emergence of ectopic motor neurons is blocked when heterologous neural crest cells were grafted into crest-ablated embryos. Experiments using the Krox20/Cre mouse line allowed the genetic tracing of the BC progeny during development. It was observed that trunk BC cells do not stay at the CNS/PNS interface. They migrate along peripheral axons and colonize the dorsal root ganglia (DRG). These data suggest that the BCs constitute a multipotent source of

PNS components, which feeds a secondary wave of emigration to populate the PNS, after the major, ventrolateral migratory stream of neural crest cells (Maro et al., 2004).

1.5 *In ovo* RNAi

In the post-genomic era, functional analysis of genes has become a rate-limiting step in the quest to answer fundamental processes. The development of high-throughput approaches has changed the way genes are analyzed. However, genome-sequencing projects as well as large-scale screens provide a tremendous amount of information about the genetic make up of an organism; long lists of genes expressed in specific tissues or distinct phases of an organism's life but little or no information about the function of the expressed proteins is provided. In order to increase the rate of functional gene analysis, new model systems are required for large-scale reverse genetic analyses and functional screens. Moreover, these model systems need to be easily accessible and efficient in producing functional read-outs of gene manipulation. Until now these criteria were met only by invertebrate systems (Adams and Sekelsky, 2002; Simmer et al., 2003); however, vertebrate systems are required for specific queries. Up to now the mouse was the animal model of choice, as a great number of genetic approaches have been well established (Jackson, 2001a; Jackson, 2001b). Unfortunately, the mouse suffers several disadvantages such as high costs for maintenance, the long time required for producing genetically modified mice and the difficulty in accessibility during development. A model organism that satisfies requirements for accessibility during development is the chicken embryo. However, the lack of genetic techniques established for this animal model restricted its use. This scenario was altered, when studies in our lab demonstrated that the combination of RNAi (Fire et al., 1998) and *in ovo* electroporation (Itasaki et al., 1999; Muramatsu et al., 1997) is a very efficient and specific tool for gene silencing in chicken embryos (Bourikas and Stoeckli, 2003; Pekarik et al., 2003). The phenomenon of RNAi (RNA interference) was discovered in *Caenorhabditis elegans*

by Fire and colleagues (Fire et al., 1998). It was observed that the response to double-stranded RNA (dsRNA) resulted in a potent sequence-specific silencing of a gene at the post-transcriptional level. In principle, RNAi is a two- step process: In the first step, the dsRNA which triggers the silencing response is cleaved into small interfering RNAs (siRNAs) of 21-23 nucleotides. This is achieved by Dicer. In the second step, siRNAs are incorporated into a targeting complex, known as RNA-induced Silencing Complex (RISC), which recognizes and destroys complementary mRNAs, thereby preventing the generation of the gene product (Meister et al., 2004). The mechanism is summarized in Fig 1.16 (Hannon, 2002).

A year before the discovery of RNAi, Muramatsu and his colleagues established *in ovo* electroporation as an efficient method of gene transfer (Muramatsu et al., 1997). The novel method of specific gene silencing *in ovo* established by (Pekarik et al., 2003), elegantly eliminates the disadvantages of the chicken embryo as a model system. Gene silencing in a temporally and spatially controlled manner allows researchers to explore diverse functions of proteins during embryogenesis (Bourikas and Stoeckli, 2003).

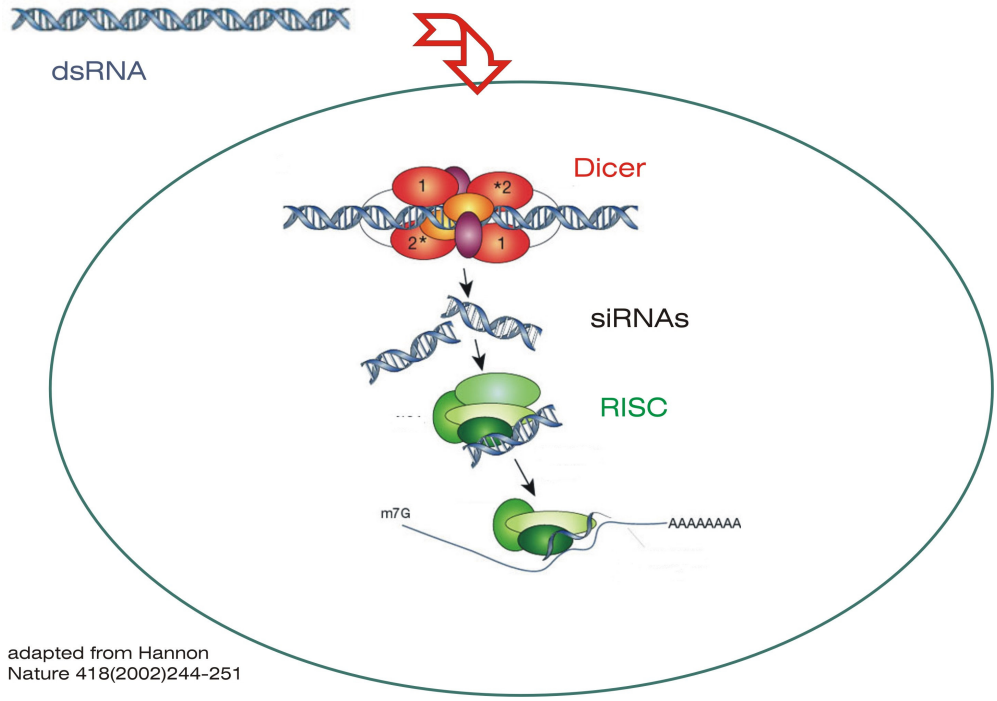


Fig: 1.16: Mechanism of RNA interference. As a first step, dsRNA is cut into 21-23 nucleotide small interfering RNAs (siRNAs) by the enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases. In the effector step, the siRNA duplexes are unwound in an ATP-depending manner and bind to a nuclease complex to form the RNA-induced silencing complex, or RISC. The active RISC then targets the complementry sequence of transcripts by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA.

1.6 Hypothesis and aim of the dissertation

SEMA6A was discovered in a screen carried out in our lab for identified postcommissural axon pathfinding. The expression of SEMA6A in the BC cells of the developing chicken spinal cord in addition to the ventral ventricular zone was very intriguing. Its presence at the CNS/PNS interface suggested a role for this gene in gatekeeping in this junction. This thesis aimed at elucidating the function of Sema6A in the developing chicken spinal cord. In addition, I also investigated the function of Sema6D in the embryonic chicken spinal cord due to its presence in the BC cells.

Chapter 2:

Results:

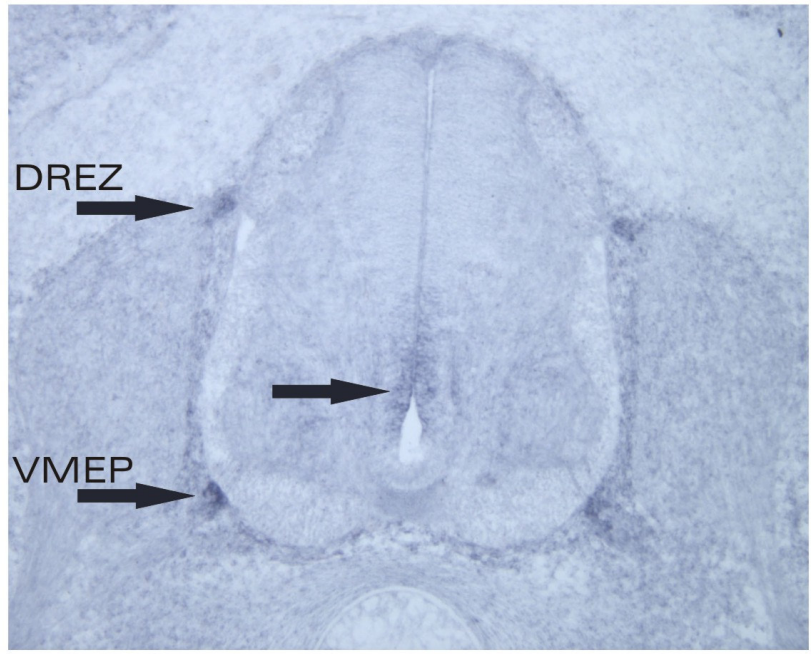
2.1 Semaphorin 6A- Gate keeper of the developing chicken spinal cord

2.1.1 Identification of chicken Sema6A and analysis of its expression

A cDNA fragment of SEMA6A was found as a candidate gene in a screen based on subtractive hybridization. The screen was carried out to identify molecular cues responsible for the rostral turning of postcommissural axons along the longitudinal axis after crossing the midline in chicken spinal cord (Bourikas et al., 2005). In a first step, candidate genes were analyzed for their temporal and spatial expression pattern. In situ hybridization analysis of SEMA6A showed an interesting expression pattern (Fig 2.1). The mRNA was expressed adjacent to the dorsal root entry zone (DREZ) and the ventral motor exit point (VMEP). The only other area of expression was the ventral ventricular zone in the developing chicken spinal cord. The DREZ and VMEP are the interface areas of the central and peripheral nervous systems (CNS/PNS). The restricted expression pattern of SEMA6A in these border areas was very intriguing and therefore this gene was chosen for further analysis. Since we had only retrieved a cDNA fragment of SEMA6A in our screen, we decided to clone the full-length cDNA. To that end, a λZAP library from embryonic chicken brain at stage 40 was screened. A 4kb fragment was obtained which turned out to be 82% and 92% identical to mouse Sema6A and human Sema6A, respectively.

•

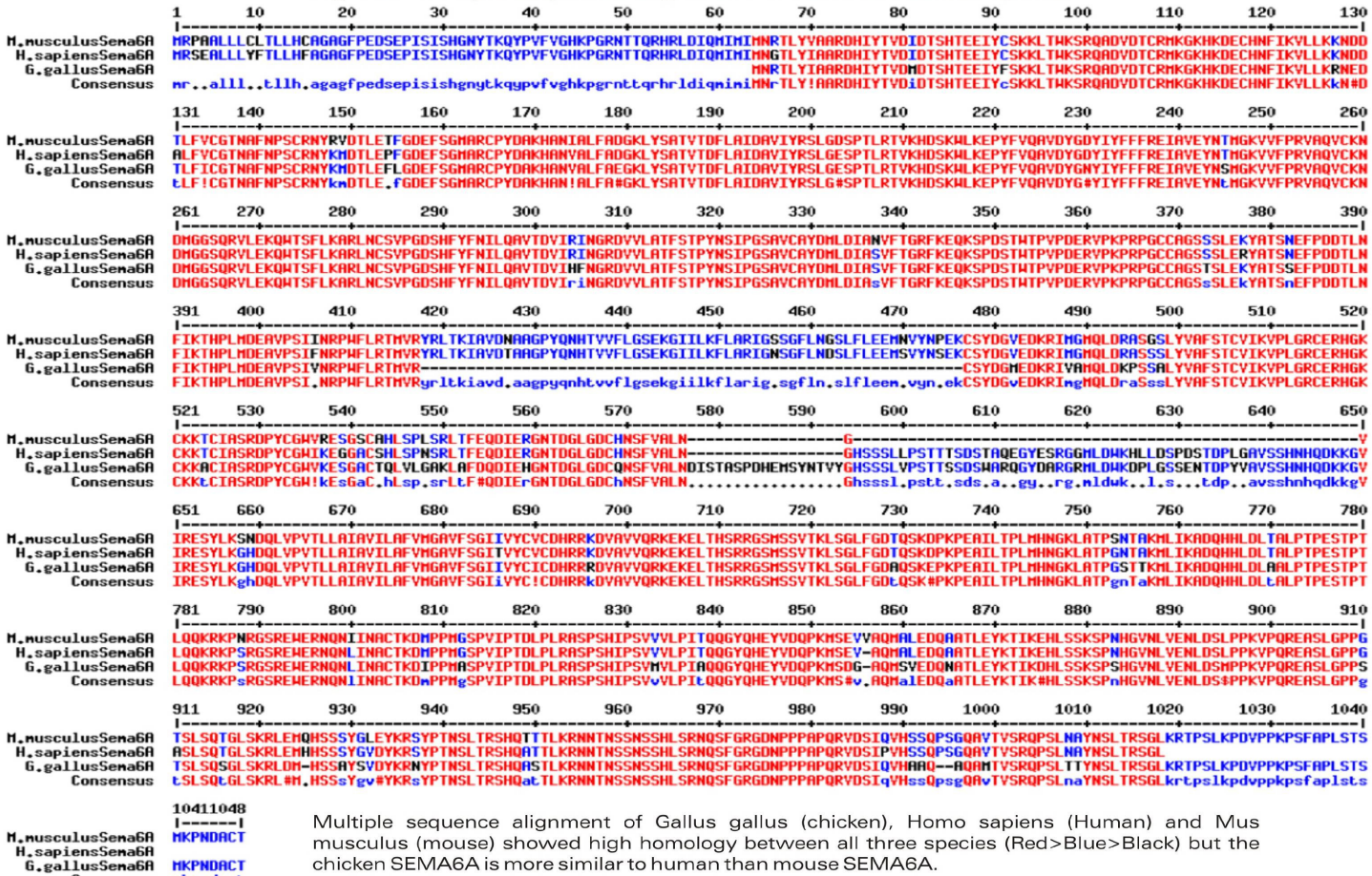
Fig 2.1: Expression of SEMA6A



SEMA6A is expressed near the dorsal root entry zone (DREZ), ventral motor exit point (VMEP) and in the ventral ventricular zone (Miglino N, Diploma Thesis, 2000).

Sequence alignment between the amino acid sequences of chicken, mouse and human Sema6A showed higher homology between human and chicken Sema6A (Fig 2.2). In collaboration with Joelle El Gemayel and Matthias Gesemann (Gemayel et al., unpublished), additional members of the chicken Sema6 family were identified, namely SEMA6B and SEMA6D. Sequence comparison for chicken Sema6 members revealed high homology only in the Sema domain (Fig 2.3).

Fig 2.2: Multiple sequence alignment of SEMA6A across species



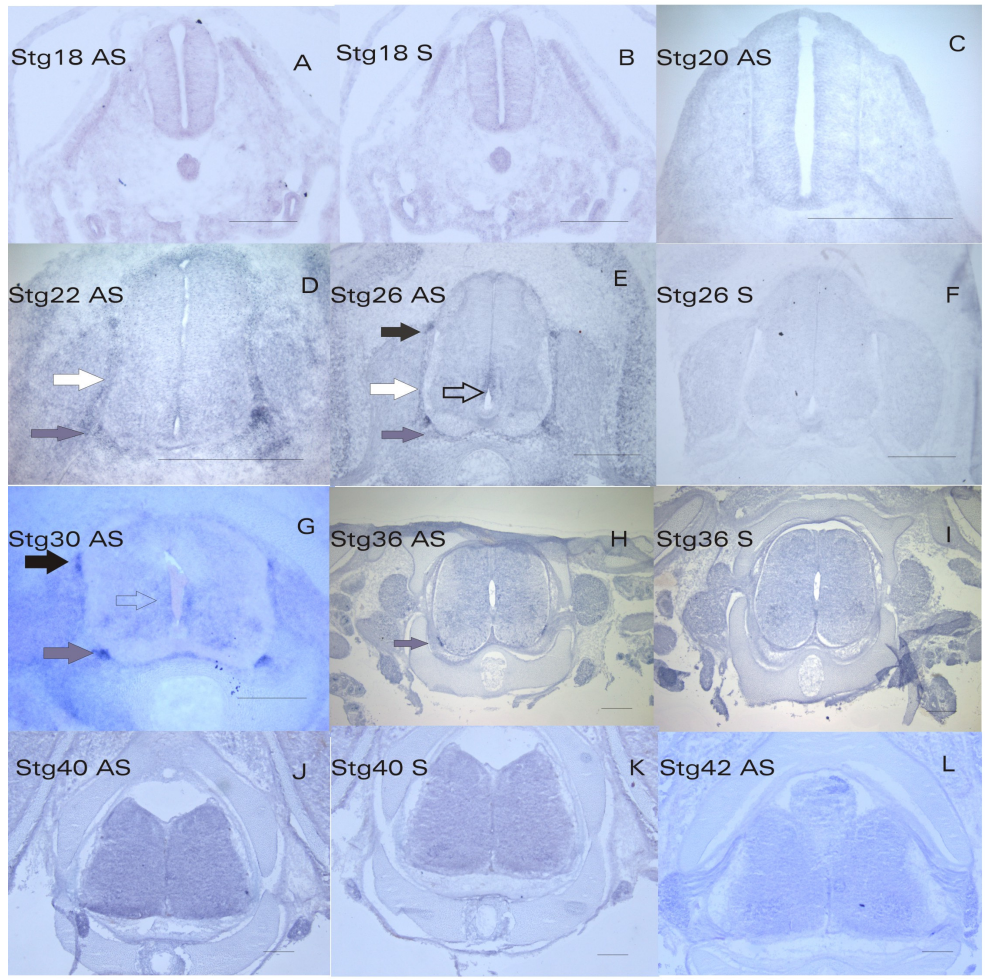
Multiple sequence alignment of Gallus gallus (chicken), Homo sapiens (Human) and Mus musculus (mouse) showed high homology between all three species (Red>Blue>Black) but the chicken SEMA6A is more similar to human than mouse SEMA6A.

Fig 2.3: Multiple sequence alignment of all the class 6 Chicken Semaphorins

Sequence alignments of all class 6 semaphorins from Gallus gallus (Chicken) showed highest homology (Red> Blue> Black) in the Sema domain (between asterisks) as expected. SEMA6B was more similar to SEMA6A than SEMA6D.

- Expression of chicken SEMA6A in spinal cord, cerebellum and retina

Fig.2.4:Expression of Sema6A in the developing chicken spinal cord



Sema6A expression in the chicken spinal cord starts around stage22 (D).There is no expression in stage 18 (A&B) and stage 20 (C). At Stage22 (E&F), the expression is seen in the migrating cells (D-white arrow) along the neural tube and also in the cells aggregating at the ventral motor exit point (VMEP) (D, purple arrow). By stage 26 expression is seen near both the dorsal root entry zone (DREZ)-black arrow and the VMEP (purple arrow, E&F). Sema6A expression is also seen in the ventral ventricular zone (E, open arrow) at stage 26. This expression is maintained till stage 30 (G).By stage 36 the expression is only at the VMEP (H&I). In older stages (J-L), no significant expression is observed.

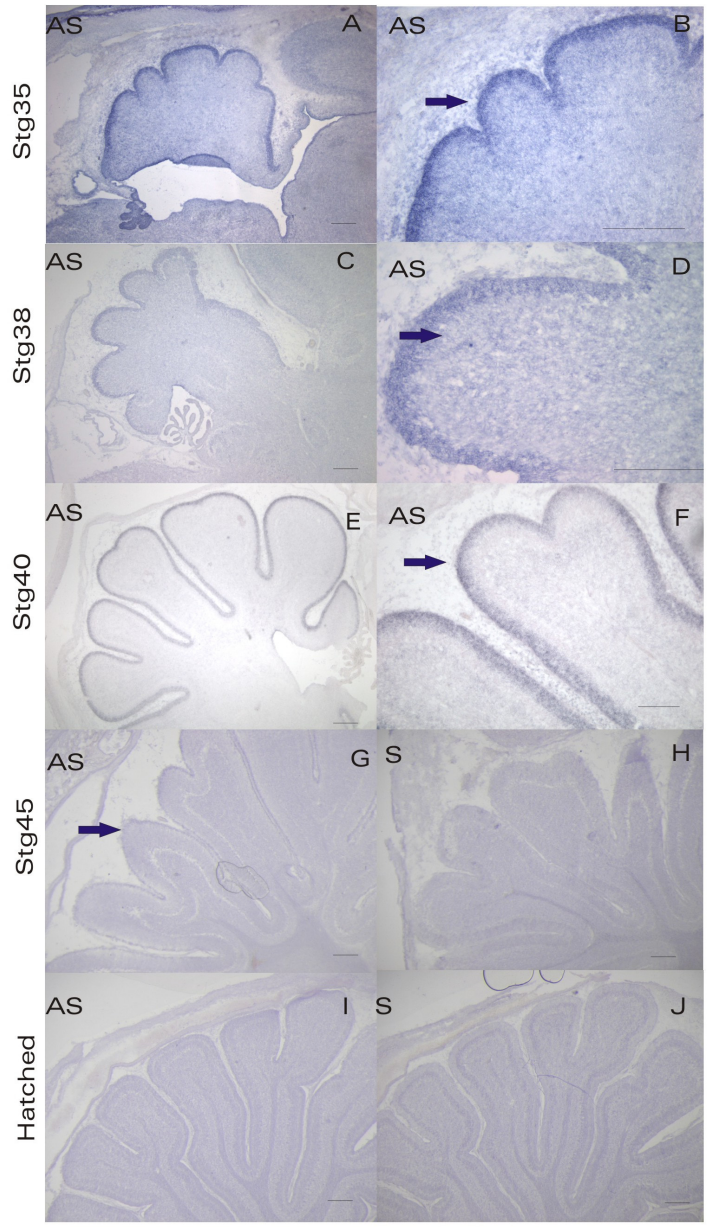
As mentioned above, SEMA6A has an intriguing expression pattern in the developing chicken spinal cord (Fig 2.4). It came up first in cells migrating alongside the neural tube and in aggregating boundary cap (BC) cells at the VMEP around stage 22 (Fig 2.4D). By stage 26, the mRNA transcripts were seen in the BC cells near the DREZ and the VMEP and in the ventral ventricular zone (Fig 2.4E&F). The expression pattern was sustained in the BC cells near the DREZ, VMEP and in the ventral ventricular zone till stage 30 (Fig 2.4G). At stage 36, Sema6A expression was present only at the VMEP (Fig 2.4H&I). At later stages,

expression in the BC cells and in the ventral ventricular zone disappeared (Fig 2.4J-L).

During the development of the chicken cerebellum, SEMA6A expression came up in the external granular cell layer at around stage 34 (data not shown). When the granule cells migrate centrally to form the inner granular layer, the external granular layer gradually thins out. Accordingly, Sema6A expression being limited to this layer decreased (Fig 2.5A-J). By stage 45, SEMA6A was no longer expressed in the cerebellum (Fig 2.5G&H). There was no SEMA6A expression in the cerebellum of hatched chickens (Fig 2.5I&J).

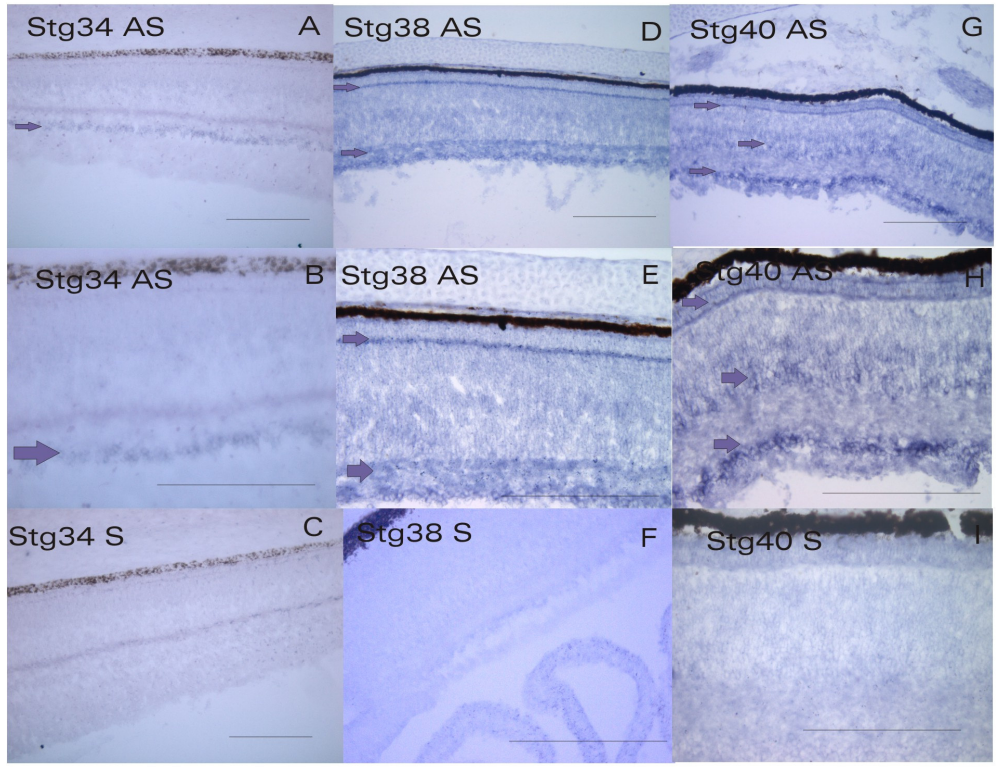
In the developing retina, SEMA6A mRNA transcripts were found in the retinal ganglion cell layer (RGCL) and in a few cells of the inner nuclear layer (Fig 2.6). Expression first came up around stage 34 in the retinal ganglion cell layer (Fig 2.6A-C). SEMA6A expression increased in the RGCL around stage 38 (Fig 2.6E) and persisted till stage 40 (Fig 2.6I-L). At stage 40, SEMA6A expression was seen in a few cells scattered in the INL (Fig 2.6H) and was still strong in the RGCL.

Fig2.5: Expression of SEMA6A in the developing chicken cerebellum



SEMA6A expression started coming up in the external granular layer of the cerebellum from stage 35 (A&B). The expression persisted at stages 38 and 40 (C-F) The expression gradually decreased as the cells in the external granular layer migrate and form the inner granular cell layer (G-J). Figs: H J are sense controls. Comparing the expression at stages 35 to 40 (A-F) with the expression in stages 45 (G&H) and hatched (I&J), there is no expression in the external granular layer in the hatched chicken cerebellum.

Fig.2.6: Expression of SEMA6A in the developing chicken retina



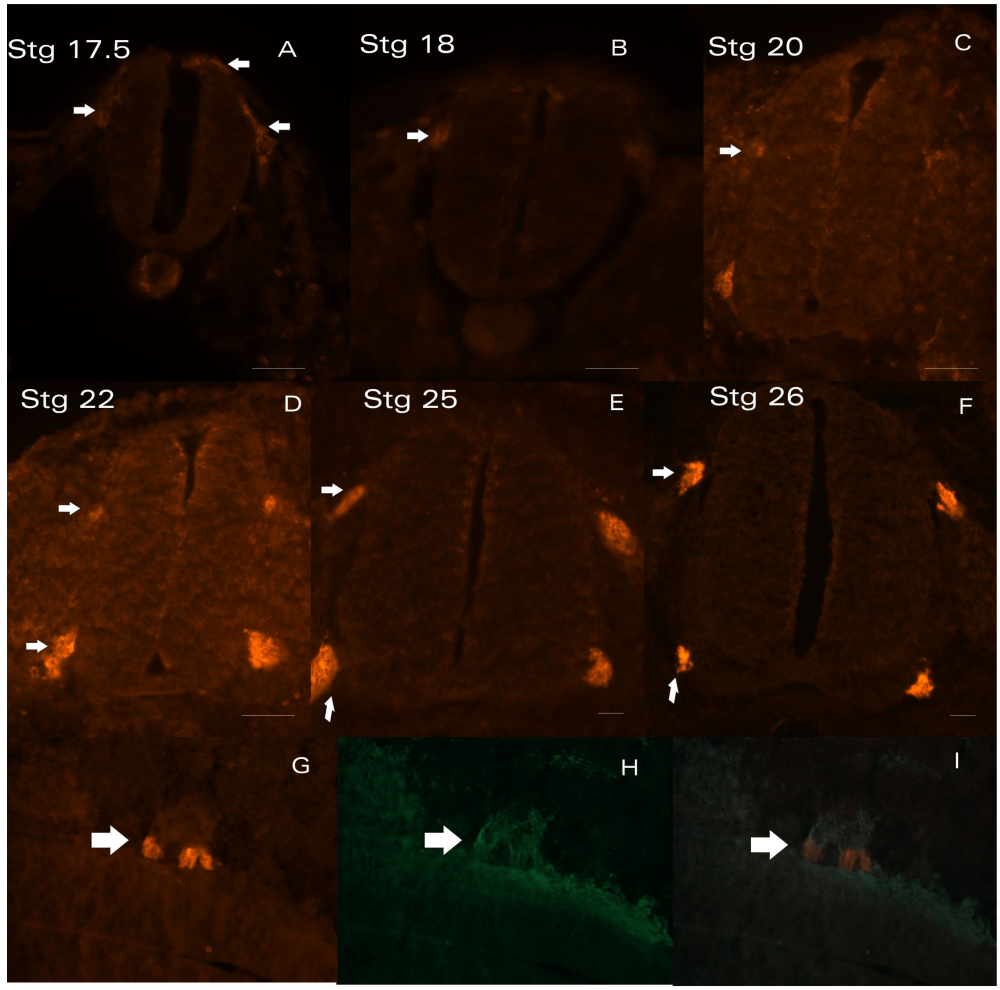
Sema6A is expressed in the developing chicken retina in the retinal ganglion cell layer (RGCL) and in few cells of the inner nuclear layer (INL) (A-I). The expression comes up in the RGCL faintly at stage 34 (A-C). At stage 38 (D-F), the expression in the RGCL is slightly upregulated and is persistent at stage 40 with additional expression in few scattered cells in the INL (G-I).

2.1.2 SEMA6A is expressed in the boundary cap cells of the chicken spinal cord

- The restricted expression pattern of SEMA6A adjacent to the DREZ and at the VMEP suggested expression in boundary cap cells (Golding and Cohen, 1997; Niederlander and Lumsden, 1996). So we searched for molecular markers for these border control or boundary cap (BC) cells. Boundary cap (BC) cells are a subpopulation of neural crest cells that migrate ventrally alongside the neural tube, and stop at entry and exit sites of the spinal cord (Golding and Cohen, 1997). These clusters of cells separate the CNS

•

Fig 2.7: 1E8 labels boundary cap cells



1E8 is a marker for precursors of Schwann cells. The sub-population of neural crest cells which form the boundary cap (BC) cells next to the dorsal root entry zone (DREZ) and the ventral motor exit point (VMEP) is stained with 1E8. At around stage 17.5 these cells are still in a migratory phase (A). They first start aggregating at the DREZ at stage 18 (B) and then at the VMEP at around stage 20 (C) and this process goes on at stage 22 (D). The BC cells ultimately occupy the entry and exit points of the spinal cord around stage 25 (E) and are still present at stage 26 (F). BC cells (G) are localized exactly where sensory axons, visualized with anti-neurofilament antibodies (H), enter the dorsal spinal cord. (I) 1E8-positive BC cells and sensory axons merged.

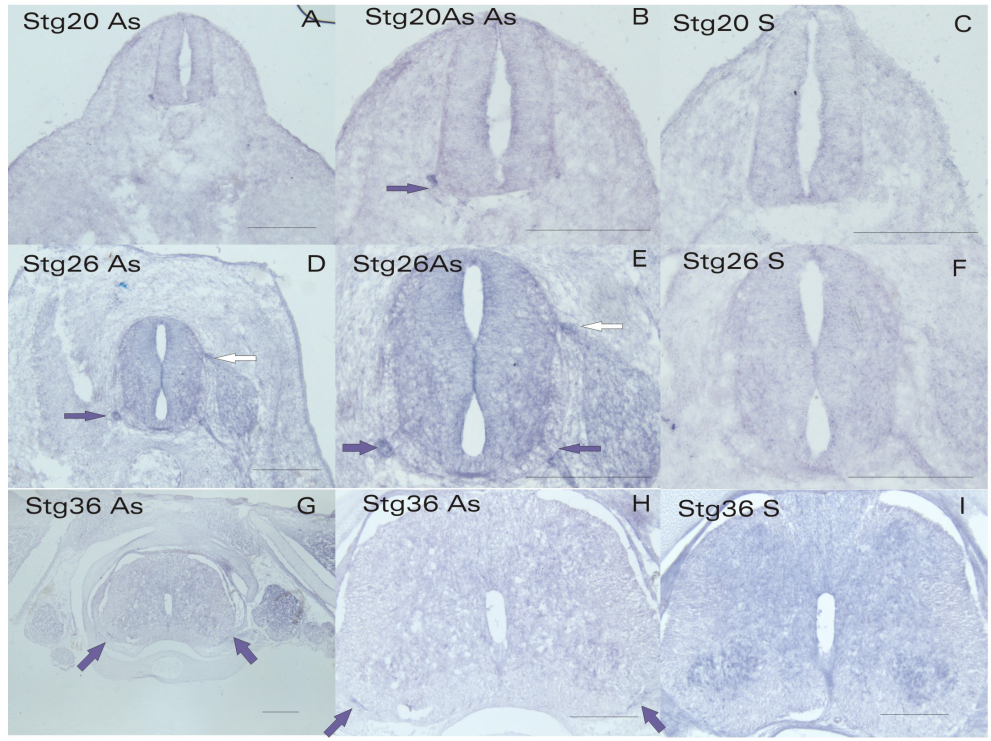
• from the PNS at the spinal cord level. Motor neurons stay within the spinal cord and extend axons that pass through the VMEP (Vermeren et al., 2003). 1E8 is a chicken-specific antibody that stains P0, a protein produced by Schwann cells and BC cells. Therefore, 1E8 is also associated with dorsal roots and ventral roots and with extending nerve trunks in older stages of chicken embryo. It stains a subset of neural crest-derived cells which are precursors of Schwann cells (Bhattacharyya et al., 1991). Neural crest cells which migrate alongside

the neural tube and ultimately cluster at the DREZ and the VMEP showed immunostaining for 1E8 (Fig 2.7A-F). At around Stg 17.5, these cells were still in the migratory phase (Fig 2.7A) followed by aggregation at the DREZ and VMEP by stage 20 (Fig 2.7B&C). Sustained 1E8 expression was seen in the BC cells from stage 22 to stage 26 (Fig 2.7D-F). At these stages, staining for 1E8 was reminiscent of the SEMA6A localization. The expression of 1E8 was found to be present at the points where the dorsal roots enter the spinal cord (Fig 2.7G-I).

2.1.3 Expression of KROX20- a known marker for boundary cap cells

- In order to confirm the localization of SEMA6A in boundary cap cells with an additional marker, we used KROX20. KROX20 is a zinc-finger transcription factor, known to be expressed in BC cells (Aquino et al., 2006; Golding and Cohen, 1997; Jungbluth et al., 2002; Maro et al., 2004). In the developing chicken spinal cord, KROX20 expression was first seen at the VMEP around stage 20 (Fig 2.8A-C). By stage 26, the expression was seen in the BC cells at both the DREZ and the VMEP (Fig 2.8D-F). At stage 36, KROX20 expression was only very faint in BC cells at the VMEP (Fig 2.8G-I). No staining was found in the dorsal BC cells. The expression of both KROX20 and the 1E8 epitope in the same areas as SEMAa6A indicated that SEMA6A was expressed in this highly specialized subset of cells called BC cells.
-

Fig 2.8: KROX 20 expression in chicken spinal cord

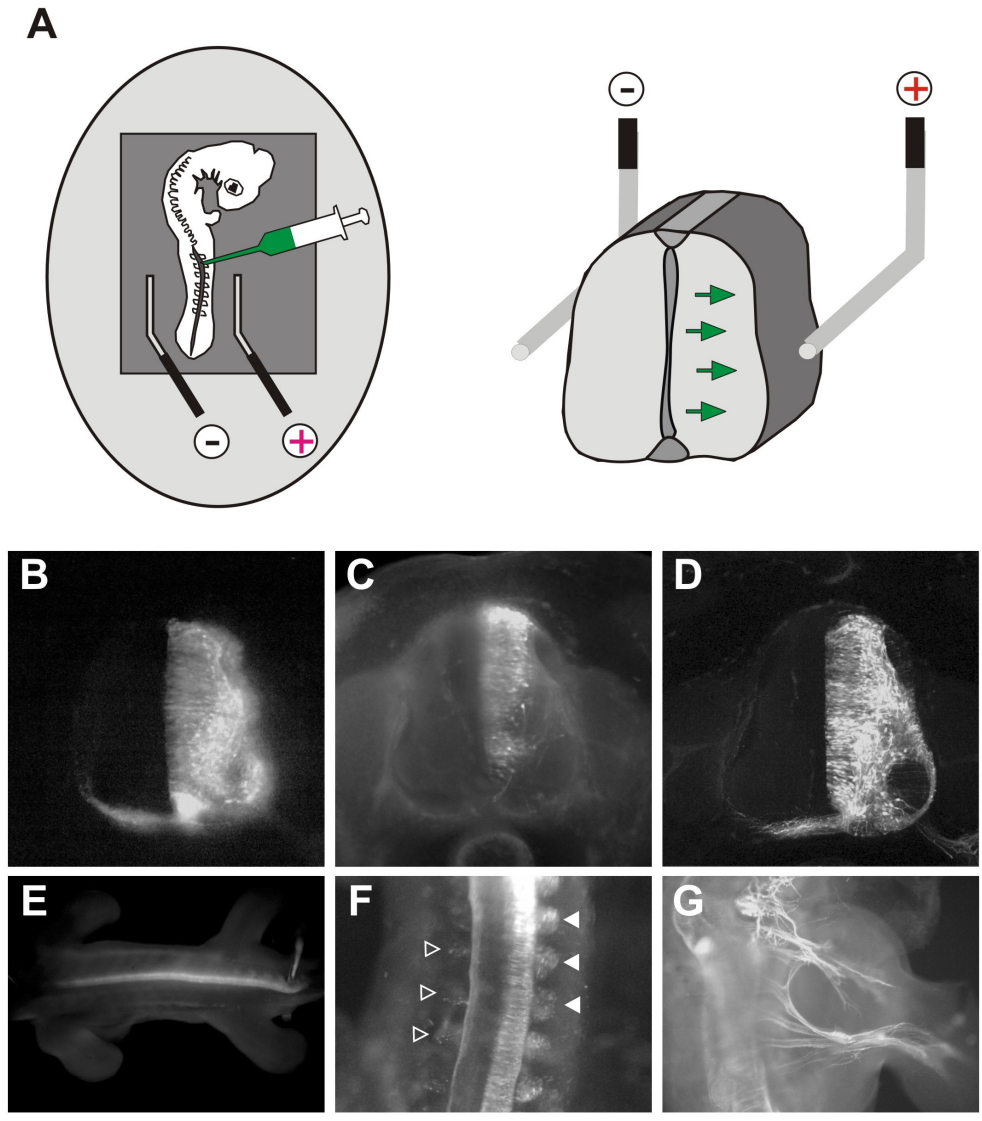


KROX 20 expression starts in the spinal cord at stage 20 (A-C). The expression is first seen in the ventral motor exit point (VMEP) (A&B). By stage 26, KROX 20 expression is seen near the dorsal root entry zone (DREZ) (white arrow) and the VMEP (purple arrow) (D-F). By stage 36, the expression is mainly near the VMEP (G-I).

2.2 SEMA6A is required for the regular arrangement of dorsal roots and the confinement of motor neurons to the spinal cord

In order to study the functional relevance of this specifically localized expression of SEMA6A, *in ovo* RNA interference (RNAi) - a novel functional tool developed in our lab-was used (Pekarik et al., 2003). This method efficiently combines *in ovo* electroporation with RNAi to

Fig. 2.9: In ovo RNAi -A tool for investigating gene function



The dsRNA derived from the gene of interest together with a YFP-expressing plasmid was injected into the central canal (A) and an electric field was applied (B). Specific areas of the spinal cord were targeted by changing the position of the electrodes and the stage of injection (B-F). Injecting at stages 14-16 resulted in transfected cells on both sides of the spinal cord (F) compared to older stages (B-D) where only one side gets transfected. The transfected cells are found also in the area of the hindlimb plexus (G) (Adapted from Pekarik et al., 2003 and Bourikas et al.,2005b)

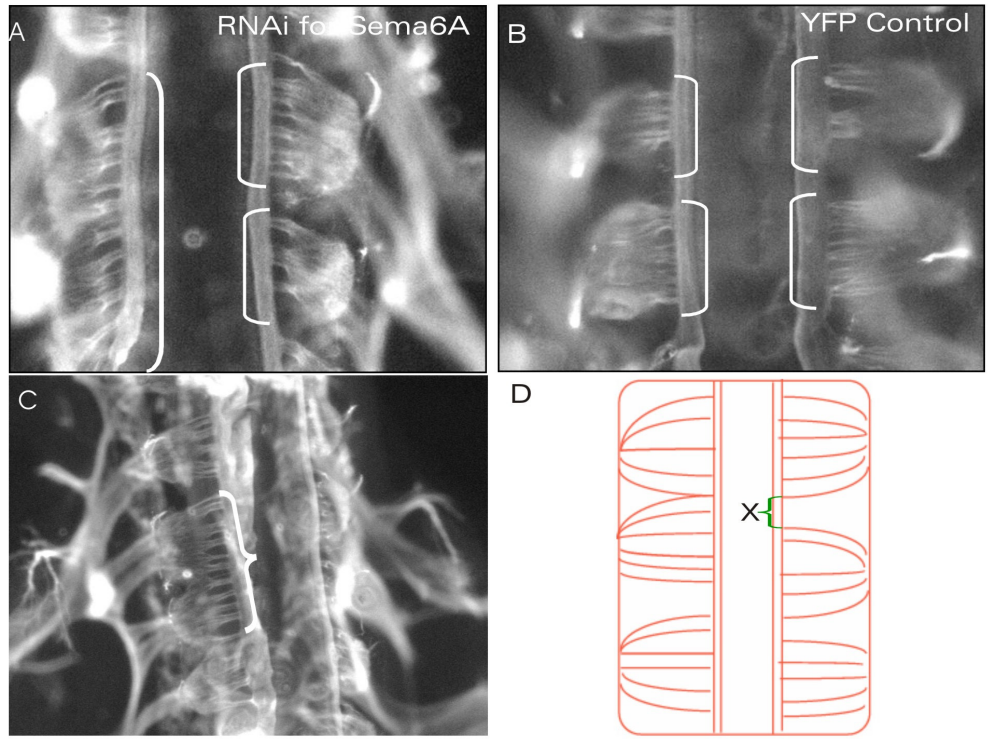
specifically silence a target gene. DsRNA of SEMA6A was generated using the same vector that we had used for the generation of the in situ RNA probes. Both strands were transcribed and annealed *in vitro*. The dsRNA was injected into the central canal of the spinal cord *in ovo* in a manner similar to the one described previously (Perrin and Stoeckli 2000). An electric field was applied in order to target the injected dsRNA into the selected area of the neural tube (Pekarik et al., 2003) (Fig 2.9). A reporter plasmid encoding YFP was co-injected with the

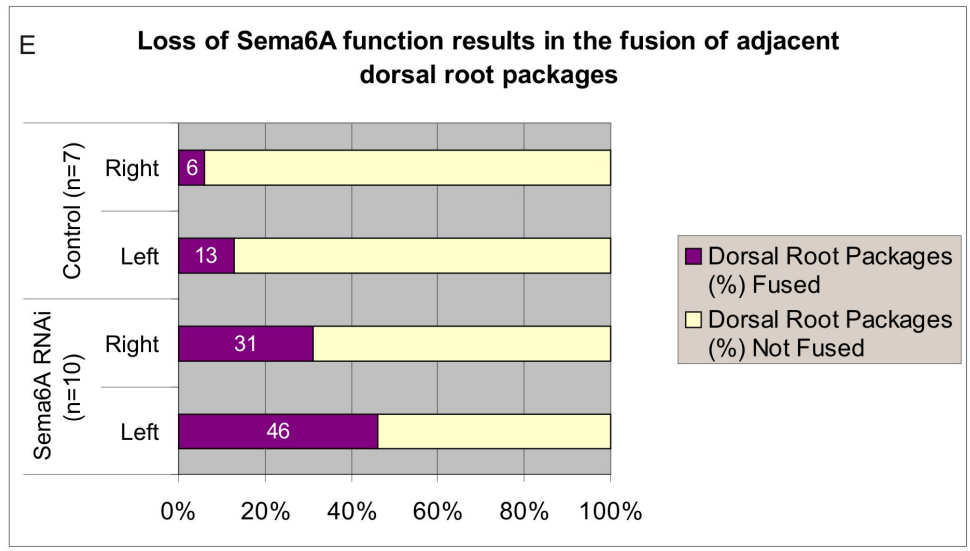
dsRNA to verify the efficiency of the injection and to trace the cells that have received the dsRNA. Embryos were injected at stages 14-16 to ensure efficient transfection of neural crest cells. In contrast to later injections, injections at these early stages resulted in the transfection of pre-migratory neural crest cells eventually found on both sides of the spinal cord (Fig 2.9G). Since the cells on both sides of the spinal cord were taking up dsRNA, age-matched embryos injected only with the YFP-expressing plasmid were used for controls. The effect of silencing SEMA6A, was analyzed in embryos sacrificed at stages 25-26 and stained with anti-neurofilament antibody as whole mounts.

2.2.1 Loss of SEMA6A function leads to fusion of dorsal roots

- In the absence of Sema6A the regular arrangement of dorsal roots was disturbed (Fig 2.10). In control embryos dorsal roots were evenly spaced (shown by short brackets -Fig 2.10B). After silencing SEMA6A (Fig.2.10A&C), the arrangement of the dorsal roots was no longer regular and they failed to segregate. Thus, loss of Sema6A function seemed to affect the way how dorsal roots enter the spinal cord. To quantify the effect, the distance (Fig 2.10D) between two adjacent dorsal root groups (i.e. dorsal roots derived from two adjacent ganglia) was measured (Fig 2.10E). Aberrant spacing of dorsal roots was found in 6 and 13% of the segments in control group but in 31 and 46% of the segments of the experimental group depending on whether the left or the right side of the embryo was analyzed.

Fig.2.10:Loss of Sema6A function results in the fusion of neighboring dorsal root packages



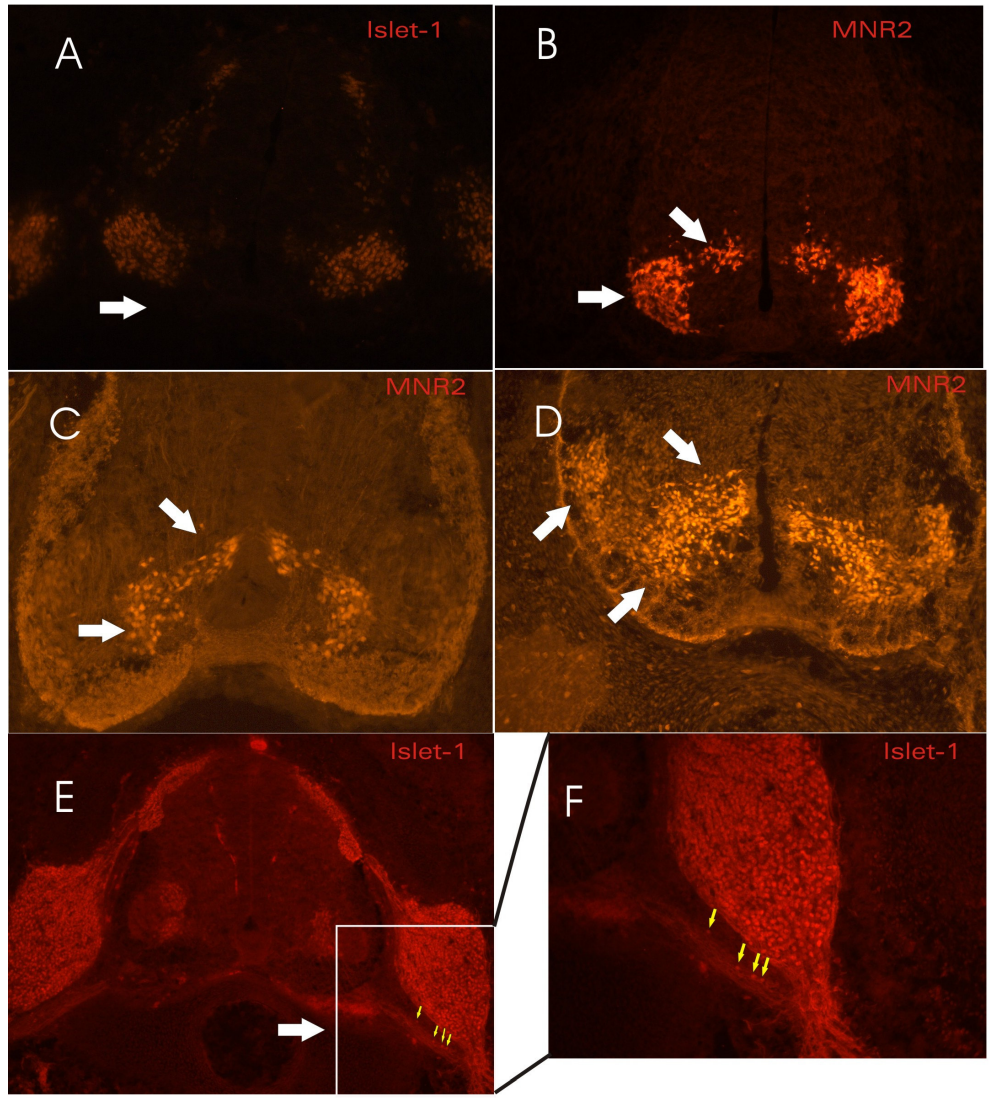


Downregulation of Sema6A led to the fusion of neighboring dorsal root packages (A&C) in the chicken spinal cord when compared to control embryos (B). The high incidence of fusion of adjacent dorsal root packages in embryos lacking Sema6A is quantified in (E).

- 2.2.2 Silencing SEMA6A leads to motor neuron emigration from the spinal cord into the periphery

- Targeted ablation of BC cells in the mouse embryo using a knockin of the gene encoding the diphtheria toxin A subunit to the KROX20 locus resulted in cell bodies of motor neurons migrating into the periphery with no significant effect on motor axon outgrowth (Vermeren et al., 2003). Since chick SEMA6A seemed to colocalize with KROX20 (Fig 2.4 and 2.8), we decided to check the effect of silencing chick SEMA6A on the integrity of the spinal motor columns. Islet-1, an antibody that stains motor neurons and sensory neurons in the DRG was used as one of the markers (Fig 2.11A). MNR2 was used as a motor neuron-specific marker to determine the effect of loss of Sema6A function on motor columns (Fig 2.11B &C). In transverse sections of control embryos, the motor neurons stayed inside the motor column of the spinal cord as expected (Fig 2.11A,B&C), whereas loss of Sema6A function due to *in ovo* RNAi resulted in the presence of motor neurons along the ventral roots (Fig 2.11E&F). Islet-1-positive neurons were found outside the VMEP along the trajectory of the motor axons (Fig 2.11E&F). The changes of the motor columns were much more evident in Fig 2.11D where the MNR2-positive motor neurons were found laterally in the ventral spinal cord. Furthermore, the MNR2-expressing cells were scattered in a much larger area in the medial spinal cord in experimental compared to control embryos (Fig 2.11B&C).

Fig 2.11: Loss of Sema6A function causes translocation of motoneurons from the spinal cord into the periphery

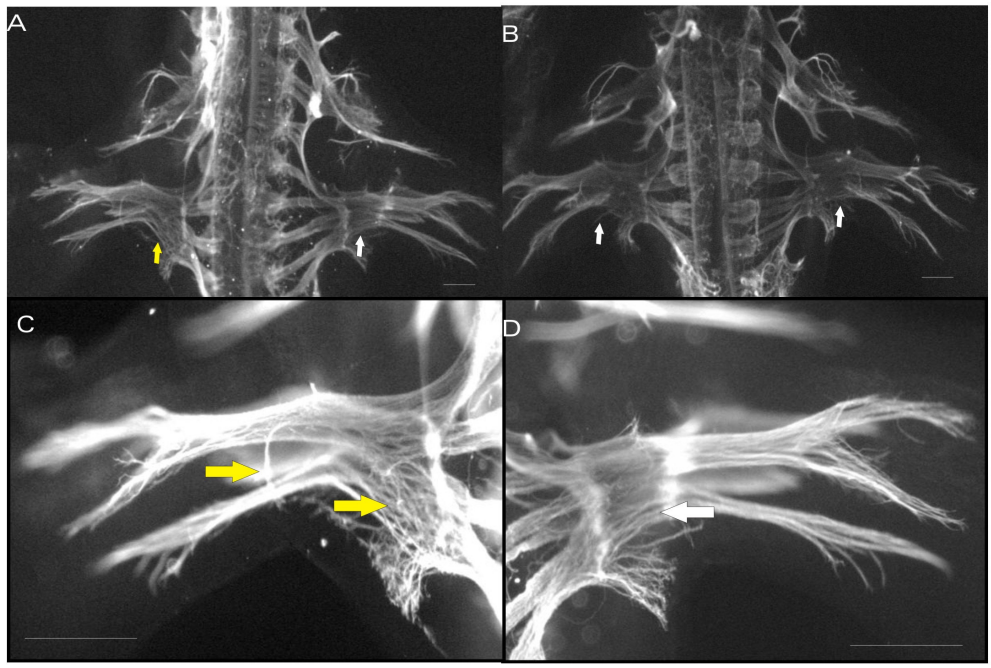


Downregulating Sema6A results in the translocation of motoneurons from the spinal cord along the motor axons into the periphery (D-F). In control embryos, Islet-1-positive motor neurons (A) and MNR2-positive motor neurons (B&C), stay inside the spinal cord. Loss of Sema6A function also leads to aberrant localization of MNR2-positive motor neurons (D) when compared to control (C). In ovo silencing of SEMA6A led to translocation of Islet-1-positive motor neurons into the periphery along motor axons (E&F).

2.2.3 Sema6A downregulation leads to defasciculation of the hindlimb plexus

In the plexus region, loss of SEM6A function resulted in defasciculation of the motor axons that innervate the limb (Fig 2.12).The arrangement of

Fig 2.12: Downregulation of Sema6A by in ovo RNAi leads to defasciculation of the hindlimb plexus



Whole-mount neurofilament staining visualized peripheral nerves in embryos lacking Sema6A (A&C) and control embryos (B&D). At higher magnification (C&D) many axon bundles crossing back and forth in the plexus can be sen in the embryo lacking Sema6A (C) when compared to the sheet-like arrangement of motor and sensory axons in the plexus in a control embryo (D). Note that the distal branches do not differ.

axons in the plexus region of control embryos was much more organized. The axons formed a sheet-like structure without any obvious axon bundles (Fig 2.12B&C). In contrast, in the embryos injected with dsRNA derived from SEMA6A, the axons in the plexus were disorganized and they formed distinct bundles that crossed back and forth in the plexus area (Fig 2.12C). The trajectories of motoneurons distal of the plexus were the same in control embryos and embryos treated with dsRNA derived from SEMA6A (Fig 2.12C&D). Therefore the phenotype could be explained by the fact that the motor neurons find themselves in an unfamiliar environment compared to their normal position in the spinal cord. Since they start

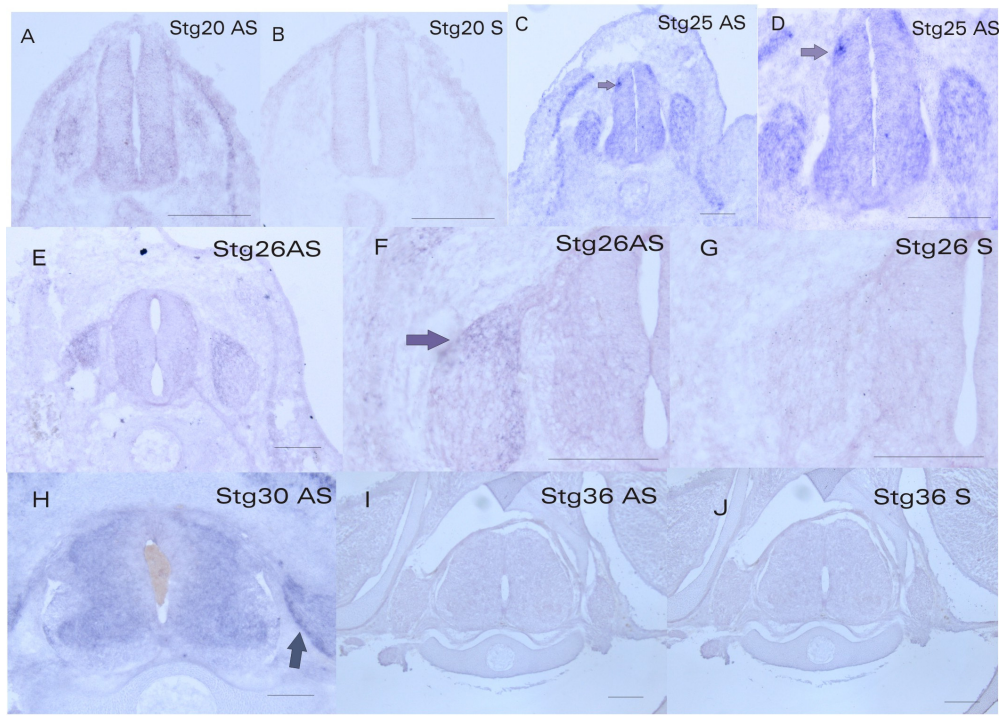
from an aberrant position they have to correct their trajectories to extend to the appropriate target muscle.

•

2.3 Comparative of expression analysis of class 6 SEMAs

In order to make sure that RNAi was specific for the targeted SEMA6 family member, we checked the expression patterns of the other 2 class 6 SEMAs namely SEMA6B and SEMA6D.

Fig 2.13: Sema6B expression in the developing chicken spinal cord



Sema6B expression starts in the spinal cord at stage 20 (A&B). There is expression in the dermamyptome faintly in the grey matter and in the dorsal root ganglia (DRGs). This expression pattern is persistent till stage 25 but additionally there is a transient expression in dorsolateral commissural neurons (C&D). From stage 26 onwards the expression in the DRGs become restricted to the anterolateral part (E-G).At stage 30 expression comes up in the epaxial area and there is expression in the DRGs (H).By stage 36 the expression in the spinal cord is gone (I&J).

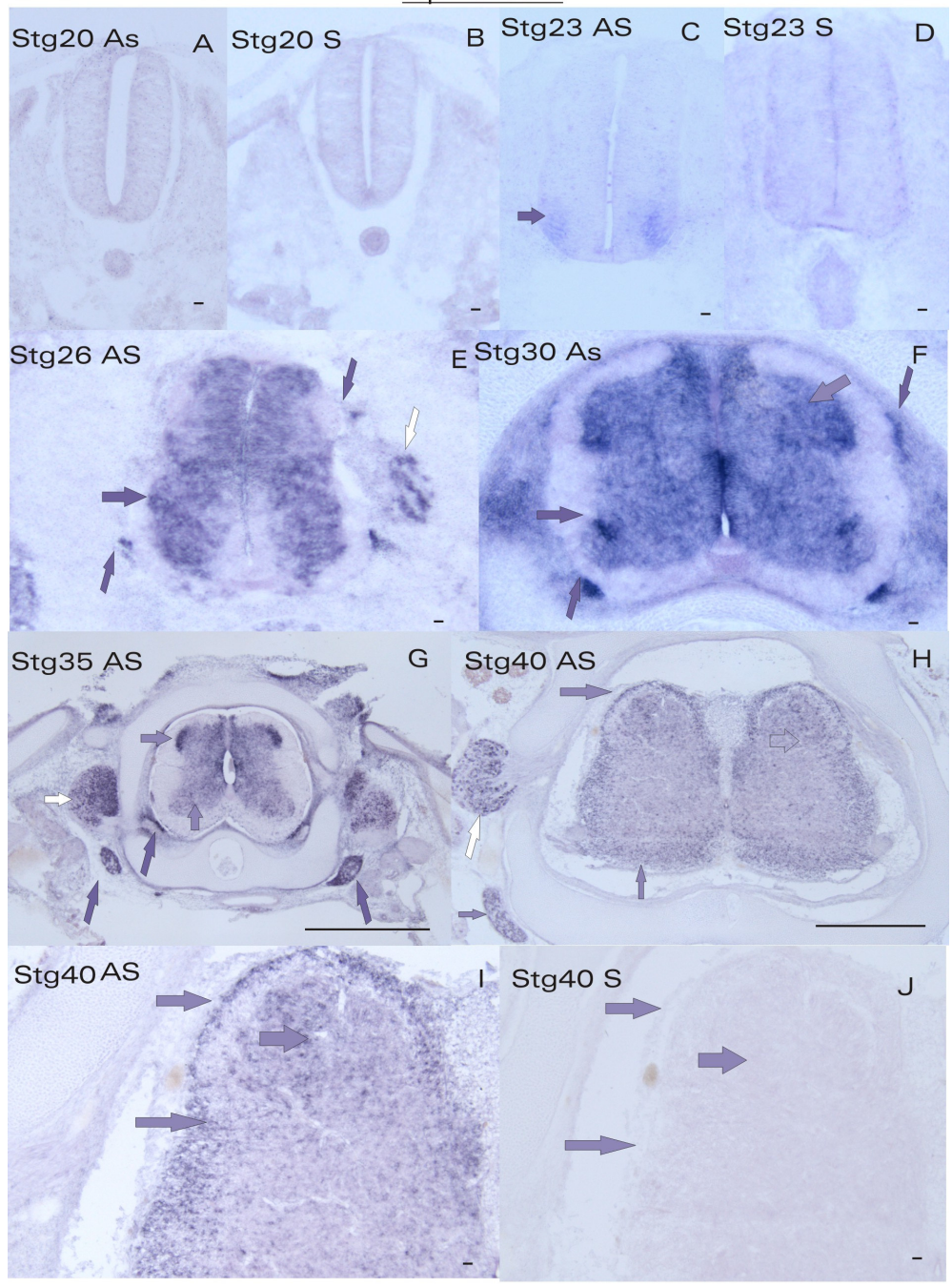
• SEMA6B expression was first seen in the chicken spinal cord around stage 20 (Fig 2.13A&B). Expression was detected in the dermamyotome and in DRGs (Fig 2.13C). The expression pattern did not change until stage 25. At that stage SEMA6B expression was transiently found in dorsolateral commissural neurons (Fig 2.13C&D). Expression in the DRGs changed drastically at stage 26 (Fig 2.13E-H). From stage 26, the expression in the DRGs became restricted to the

dorsomedial part (Fig 2.13E-H). At stage 30, SEMA6B expression was found in the epaxial muscle (Fig 2.13H). By stage 36, SEMA6B was no longer expressed in the spinal cord (Fig 2.13I&J).

- Like SEMA6A, SEMA6D was found in BC cells. However, expression was widespread. SEMA6D was also expressed in motor neurons and the DRGs (Fig 2.14). SEMA6D first came up in the motor neurons of the chicken spinal cord around stage 23 (Fig 2.14A-D). From then on, there was widespread SEMA6D expression in the gray matter (Fig 2.14E-J). At stage 26, the BC cells started to express SEMA6D mRNA. Expression in the motor neurons and the DRGs persisted until stage 30 (Fig 2.14E-H). From stage30 onwards, the expression of SEMA6D was restricted to the dorsal gray matter (Fig 2.14F-H). Lamina I of the spinal cord showed high levels of SEMA6D expression from stages 30 to 35 (Fig 2.14F&G). At later stages, SEMA6D expression was upregulated in the dorsal and ventral funiculi (Fig 2.14H-J).

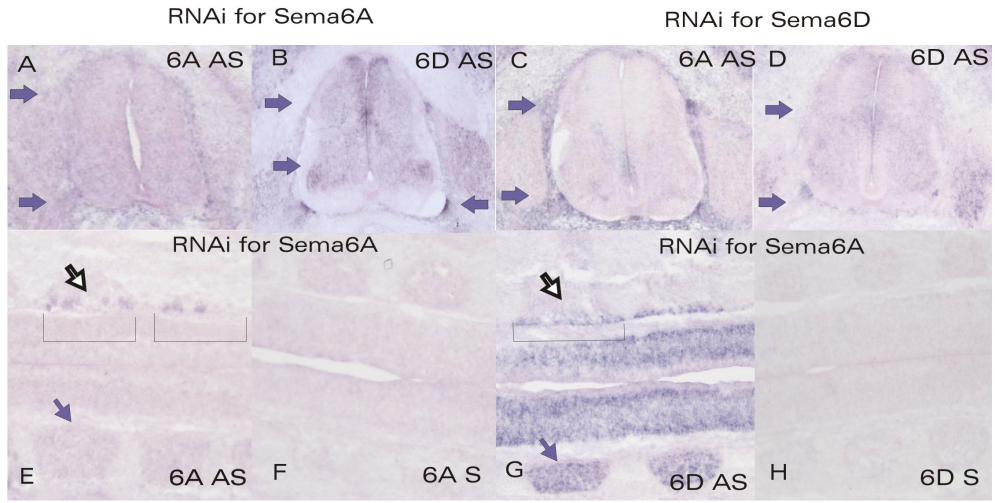
- Since both SEMA6A and 6D were expressed in the BC cells it was important to check whether their downregulation was mutually exclusive.

Fig.2.14: Expression of SEMA6D in the developing chicken spinal cord



SEMA6D was not expressed in the developing spinal cord until stage 23 (A-D). It was present in the motor neurons at this stage (C&D) and then gradually increased in the gray matter(E-J). At around stage 26 (E), the boundary cap cells started showing SEMA6D expression. At later stages, SEMA6D expression in the spinal cord started shifting into the dorsal parts (F-J). Lamina I of the spinal cord showed stronger expression than the rest of the gray matter (F & G). At stage 40 (H -J), the dorsal and ventral funiculi also showed strong SEMA6D expression along with the dorsal laminae.

Fig. 2.15:Downregulation of SEMA6A and SEMA6D is specific



Downregulating SEMA6A resulted in decreased expression of SEMA6A (A&E) while SEMA6D expression was maintained at normal levels (B&G). Silencing SEMA6D resulted in the specific downregulation of SEMA6D expression (D)while SEMA6A expression was unchanged (C). Figs F &H serve as sense controls for SEMA6A and 6D respectively. In E&G, the images show the dorsal roots at the dorsal root entry zone (DREZ) shown by white arrows while the purple arrows show the dorsal root ganglia on the other side. Loss of Sema6A function resulted in the disappearance of SEMA6A expression in some boundary cap (BC) cells at the dorsal roots (E) indicated by a white arrow.

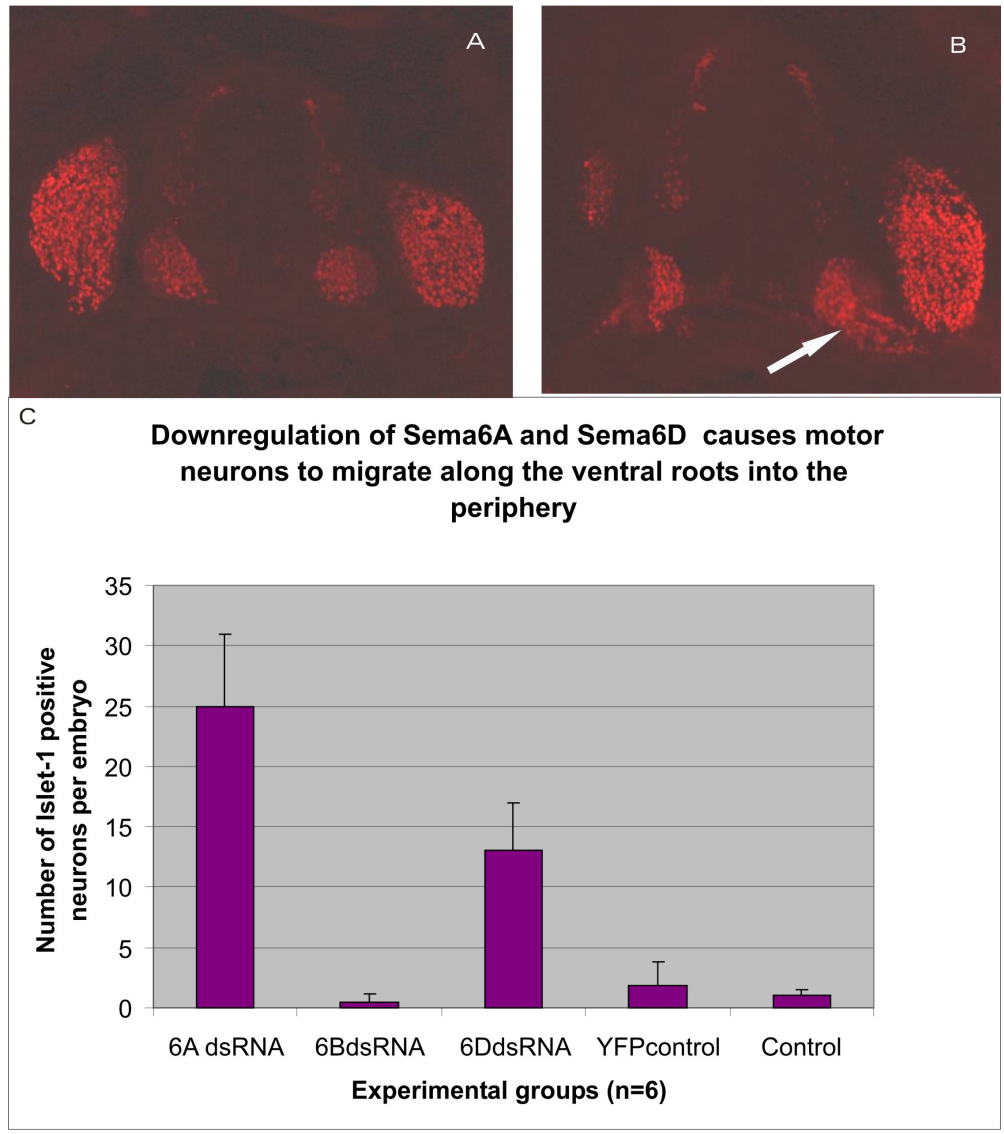
-
-

2.4 Downregulation of SEMA6A and SEMA6D by *in ovo* RNAi is specific

The concomitant expression of SEMA6A and 6D in BC cells allowed us to check the specificity of gene silencing by *in ovo* RNAi. Downregulating SEMA6A led to decreased expression of SEMA6A (Fig 2.15A, E&F) without any changes in SEMA6D expression level (Fig 2.15B, G&H). Similarly, silencing SEMA6D led to diminished Sema6D expression (Fig 2.15D) while SEMA6A expression remained unaffected. A closer look at longitudinal sections showed that SEMA6A expression was absent from BC cells present at the entry point of some dorsal roots in SEMA6A downregulated embryos (Fig 2.15E) while the expression is unaffected in SEMA6D downregulated embryos (Fog 2.15G).

2.5 Immunohistochemical analysis of motor column integrity in the spinal cord after loss of Sema6D function

Fig 2.16:Islet-1 positive motoneurons get translocated into the periphery from the spinal cord after downregulating Sema6D



Loss of Sema6D led to the translocation of Islet-1-positive motor neurons out of the spinal cord into the periphery. No motoneurons leave the spinal cord in control embryos (A). In embryos lacking Sema6D motoneurons were streaming out of the spinal cord at the ventral motor exit point (VMEP) in large numbers (arrow, B). The number of motor neurons found in the periphery was quantified. The mean values are given.

The effect of Sema6D downregulation on motor column integrity was investigated in collaboration with A. L. Daetwyler. Islet-1 was used as a marker for the analysis of the effect of loss of Sema6D function on motor neurons. Downregulating Sema6D in the developing chicken spinal cord led to the displacement of motor neurons from the spinal cord into the periphery (Fig 2.16). This phenotype was similar to the one observed after downregulating Sema6A. The number of dislocated

motoneurons was counted in embryos lacking Sema6A and Sema6D and compared to embryos without Sema6B, YFP-injected embryos and uninjected controls (Fig 2.16C). A large percentage of motor neurons were dislocated after downregulating Sema6A and Sema6D but not in control groups. Loss of Sema6B did not affect the position of motor neurons.

2.6 Conclusion

In summary, these data support a role for Sema6A as a gatekeeper at the entry and exit sites of the developing chicken spinal cord. Loss of Sema6A function led to aberrant arrangement of dorsal roots at the DREZ and to the escape of motoneurons from the ventral spinal cord at the VMEP.

Chapter 3

Discussion and Outlook

3.1 Comparison of mouse and chicken SEMA6A expression reveal some similarities and dissimilarities

The expression pattern seen in the developing chicken spinal cord was quite different from the expression seen in mouse. Sema6A in mouse was

expressed in the spinal cord and dorsal root ganglia (Suto et al 2005; Klostermann et al, 2000; Zhou et al., 1997 and Xu et al 2000). The expression was stronger in the ventral spinal cord and all the somites during early phases of development (Xu et al., 2000). SEMA6A expression in the chicken embryo started in the BC cells and the ventral ventricular zone and at later stages it was present only at the VMEP. Major differences are the absence of expression in BC cells in mouse and the strong expression of SEMA6A in the DRGs in the mouse that is not seen in chicken DRGs. Therefore, SEMA6A may function, at least in part in a species-specific manner.

Comparison of the SEMA6A expression pattern in the developing chicken and mouse cerebellum revealed significant differences (Zhou et al., 1997 and Kerjan et al., 2005). High levels of SEMA6A transcripts were found both in the inner external germinal layer (EGL) and in the inner granular layer of the mouse cerebellum. In the chicken cerebellum, SEMA6A expression was high in the external germinal layer but absent in the inner granular layer. This expression did not reflect the distribution of Sema6A protein in the mouse cerebellum. Kerjan and colleagues have shown that though SEMA6A mRNA is present in the IGL, there is a conspicuous absence of the Sema6A protein in this area. This suggests that radially migrating cells in the molecular layer and postmigratory granule cells in the IGL no longer express Sema6A protein although they still stain positive for mRNA when analyzed by in situ hybridization (Kerjan et al., 2005). Due to the unavailability of chicken-specific Sema6A antibodies, this aspect has not yet been investigated. With the expression pattern observed in chicken cerebellum, it seems that Sema6A is important for the initiation of the migration of the granular cells from the outer EGL into the inner EGL and the IGL.

Despite the differences, there are similarities in expression between the mouse and chick especially in the developing retina (Xu et al., 2000 and Zhou et al., 1997). The presence of SEMA6A mRNA in the retinal ganglion cell layer and the inner nuclear layer is seen both in the chicken and mouse

retinas. The outer and inner segments of the photoreceptor layer also show a bit of SEMA6A expression in the chicken retina.

3.1 Silencing either SEMA6A or SEMA6D induce an effect similar to that of BC cell ablation

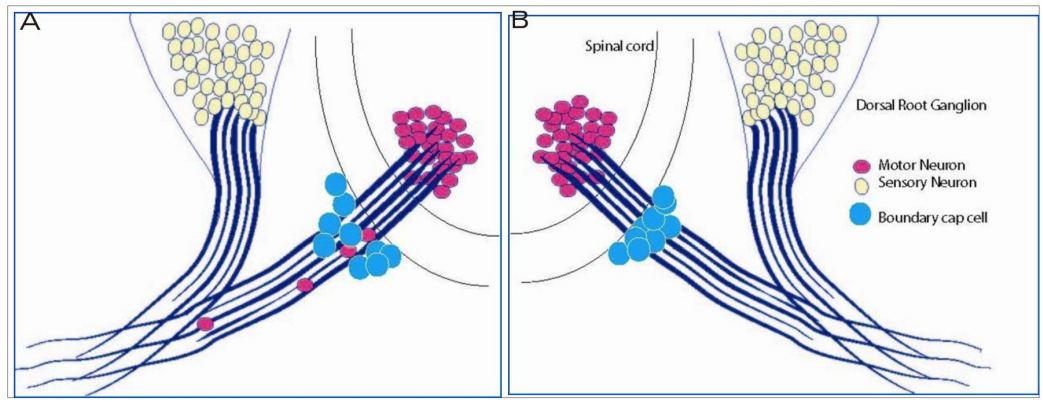
The striking expression patterns of SEMA6A and 6D led to speculation about their function. While SEMA6D was expressed in motoneurons as well as in sensory neurons, high mRNA levels for SEMA6D and SEMA6A were detected in the BC cells.

Vermeren and colleagues have shown that selective ablation of BC cells in the mouse embryo using a knockin of the gene encoding the diphtheria toxin A subunit into the Krox20 locus resulted in the emigration of motor neurons out of the spinal cord (Vermeren et al., 2003). Comparing the phenotype generated by ablating BC cells and the one generated by just silencing SEMA6A or SEMA6D, suggests a very important role for these two Sema6 members in keeping the motor neurons inside the spinal cord. The loss of SEMA6A and SEMA6D resulted in the translocation of motor neurons along their axons into the periphery in chicken embryos similar to the migration of motor neurons observed in mice after genetical ablation of BC cells.

The fact that SEMA6A and SEMA6D transcript upregulation occurs only after neural crest cells have initiated their migration suggests that Sema6A and 6D are not required during early events of boundary cap cell development. Furthermore, transcript levels for both molecules are strongly increased by the time these cells aggregate in the area of the ventral and dorsal roots. Thus, it seems reasonable to speculate that Sema6A and Sema6D might be required for boundary cap cells aggregation. Thus, semaphorin 6A and 6D in boundary cap cells might act as “gate keepers” by keeping neuronal cell bodies confined within specific locations by repulsive interactions. The phenotype that was observed at the exit sites could be caused by two different reasons. Firstly, the silencing of SEMA6A

in the boundary cap cells may remove a restraining signal for motor neurons and therefore allow them to exit the spinal cord. Secondly, the loss of SEMA6A may result in aberrant functioning of boundary cap cells due to perturbation of cell-cell contacts resulting in decreased clustering (Fig 3.1). This in turn would result in looser and less fasciculated axon growth because of missing signals that define their bundling. The defasciculation in the plexus region of the hindlimb induced by SEMA6A loss of function might be explained by the fact that motor neurons which usually are confined to the spinal cord are found in the periphery. The unfamiliar environment would result in pathway corrections necessary for reaching their target muscles. The appropriate target muscles in the distal limb were still contacted due to the presence of guidance cues (Eberhart et al., 2000; Vargesson et al., 2004; Honig et al., 2005; Eberhart et al., 2002; Lance-Jones & Dias, 1991).

Fig 3.1: Hypothetical model for the emigration of motor neurons due to loss of Sema6A or Sema6D function



In this hypothetical model, Fig B serves as a control where the boundary cap (BC) cells are clustered normally at the ventral motor exit point (VMEP) and the motor neurons are present inside the spinal cord. In contrast, loss of Sema6Aor 6D function may lead to aberrant clustering of BC cells at the VMEP (A) leading to the emigration of the spinal motor neurons into the periphery.

3.2 PlexinA2 and PlexinA4 could be potential binding partners for SEMA6A

In collaboration with Olivier Mauti, we searched for the binding partners for SEMA6A. To this end, the expression pattern for all chicken Plexins and

Neuropilins were analyzed. The presence of PLEXINA2 and PLEXINA4 in motor neurons and DRGs suggested that these two plexins could serve as binding partners for SEMA6A (Mauti et al., submitted). Suto and colleagues showed that plexinA4 bound class 6 transmembrane Semaphorins, Sema6A and Sema6B, and mediated their repulsive activities, independently of neuropilin-1. Moreover, Plexin-A4 mutant mice also showed defects in the trajectory and projection of peripheral sensory axons (Suto et al., 2005). These data suggest that Plexin A2 and A4 might act as receptors for Sema6A. Loss of function studies for PLEXINA2 and PLEXINA4 combined with receptor-ligand binding assays will reveal which Plexin is responsible for the phenotypes observed after silencing SEMA6A.

3.3 PlexinA1 could be a probable receptor for SEMA6D in chicken spinal cord

The Sema6D receptor, PlexinA1, was found to be highly expressed in developing motor and sensory neurons (Toyofuku et al., 2004b and Mauti et al., submitted). Moreover, PplexinA1 was found to bind to Sema6D and mediate its effect on neural tube formation, cardiac morphogenesis and cardiac cell migration (Toyofuku et al; 2004a and 2004b). These data suggest that PlexinA1 could be the receptor mediating the effect of SEMA6D on motor neuron migration. More functional studies have to be carried out to test this hypothesis.

3.4 Differential effect of Sema6A on the growth cones of DRG neurites and sympathetic ganglion neurites

The extracellular domain of Sema6A was subcloned by Xu and colleagues to create C-terminally tagged Myc-His or Fc fusion proteins, Sema6A-mh and Sema6A-Fc, respectively (Xu et al., 2000. Using the Sema6A-Fc construct they showed that Sema6A could induce a collapse of sympathetic growth cones at a concentration ten-fold lower than the concentration needed to collapse sensory axon growth cones). Moreover, they also

showed that Sema6A-Fc formed a dimer and this dimerization was required for the collapse of sympathetic growth cones. This effect was consistent with the presence of Sema6A in the tissue surrounding these ganglia.

Class6 semaphorins might also act as attractants for motor and sensory neurons, specifically guiding these axons towards the entry zones or exit points of the spinal cord. That semaphorins can indeed act as attractant has been shown in several recent reports (Dent et al., 2004; Wolman et al., 2004). However, only functional assays will provide evidence about the role of class 6 semaphorins in boundary cap cells.

Chapter 4

Experimental Procedures:

Model system:

Fertilized Hisex eggs were obtained from a local supplier. For *in ovo* injections, a window was cut into the eggshell after 2 days of incubation at 38.5^0C. The window was closed with a glass cover slip and sealed with molten paraffin allowing repeated opening and intervention. Chick embryos of different developmental stages (Hamburger and Hamilton, 1951) were collected, fixed in 4% paraformaldehyde, cryoprotected in 25% sucrose in 0.1M sodium phosphate buffer and embedded in OCT (Embedding medium from Tissue-Tek, Sakura) in isopentane cooled on dry ice. The embryos were sectioned on a cryostat (Leica CM1850) and 20-25µm thick sections were collected on Superfrost Plus (Menzer-Glazer) glass slides for microscopy. After drying the slides for 30 minutes at 37℃, they were stored at -20℃ till further use. In situ hybridization and immunohistochemistry was performed on these sections. Intact embryos were also collected for whole mount immunohistochemistry and whole mount in situ hybridization.

Cloning:

Cloning of Sema6A:

The 728 bp long cDNA fragment (Clone 37) obtained from the screen (Pekarik et al 2003) was cloned into pCRII-TOPO vector (Invitrogen). To get the full-length sequence of clone 37, a λZAP library from embryonic chicken brain at stage 40 was screened. The library was constructed by R. Zuellig at the University of Zurich (Zuellig et al., 1992). A 4kb fragment was obtained which on sequence analysis was found to be homologous to mouse and human Semaphorin 6A (Sema6A). The full length thus obtained was cloned into pBSKS+ vector from Stratagene. This work was carried by Nicola Miglino (Diploma thesis, University of Basel).

Cloning of Sema6B:

No hits were obtained for Sema6B in the EST database hence cDNA fragment for this gene was cloned using RT-PCR. A putative cDNA assembled based on genomic information was used as a template in order to design sense and antisense primers. Total RNA was prepared using spinal cord and DRG tissue isolated from stage 30 chicken embryos. Random and oligodT primed first strand cDNAs were generated using the SuperscriptII reverse transcription kit (Invirtogen, Carlsbad CA) according to the manufacturers' instruction. A 656 bp long fragment for Sema6B was amplified using the following two primers 5' ATCCAGCGCATCCTCAAG (sense) and 5' CCCATGTCGTTCTTGCAC (antisense). Obtained PCR fragments were cloned into the Topo TA cloning vector (Invirtogen, Carlsbad CA) between EcoRI restriction sites, and subsequently sequenced to verify the identity of the insert. This cloning was done in collaboration with Matthias Gesseman and Joelle Gemayel, Brain Research Institute, Zurich.

Sema6D cDNA:

The cDNA sequences for chicken semaphorins were assembled using the combined information from the chicken EST database prepared by BBSRC (http://www.chick.umist.ac.uk) and the chicken genomic database (http://www.ensembl.org/Multi/blastview?species=Gallus_gallus).

ChEST225N10 corresponding to Sema6D was obtained from Geneservice Ltd. Probes and dsRNA were derived from ESTs. For this purpose the

bacteria were streaked on agar plates containing carbenicillin. After incubating these plates at 37°C overnight, single colonies were picked to inoculate cultures in LB-medium containing carbenicillin. These cultures were incubated at 37°C with constant shaking. After overnight incubation, bacteria were spun down and plasmid DNA was extracted using the Macherey Nagel plasmid extraction kit. The concentration and quality of the plasmid DNA was measured using a Nanodrop spectrophotometer (ND-1000). The plasmid DNA thus obtained was sequenced in order to confirm the identity of the received EST. After confirmation, glycerol stocks were prepared and stored at -80°C. Plasmid DNA was stored at -20°C till further use.

In situ hybridization:

In situ hybridization was used to localize mRNA on 25µm thick cryostat sections or in intact embryos. The plasmids containing the relevant fragments of the genes of interest were linearized using restriction endonucleases. Linearized plasmids were DIG-labeled by incubating 2 µg of each DNA with 2 µl digoxigenin (DIG) labeling mix (Roche), 2 µl of T3, T7 or SP6 RNA polymerase (Roche), 2 µl of 10 X transcription buffer (Roche), and DEPC-treated H_2O added to a final volume of 20 µl for each reaction, at 37°C for 2 hours. After incubation, 2 units of RNase free DNaseI (Roche, 10U/µl) was added to the mix, and incubated at 37°C for 30 min, after which 2 µl of 0.2 M EDTA, pH 8.0, were added to stop the nuclease treatment. The cRNA probe was ethanol-precipitated and dissolved in 50 µl of DEPC-treated H2O.

Sema6A insitu probe preparation:

The cDNA fragment (728bp) obtained from the screen (Pekarik et al. 2003) was cloned into pSP72 vector (Promega) in order to produce antisense and sense RNA probes. The plasmid was linearized with the restriction enzymes- HindIII and Asp718. The RNA probes were digoxigenin-labeled and transcribed using T7 and Sp6 promoters. The Dig RNA labeling mix,

T7 polymerase and SP6 polymerase were obtained from Roche Applied Sciences.

Sema6B insitu probe preparation:

The plasmid (pcRII TOPO from Invitrogen) was linearized with HindIII and XbaI in order to produce antisense and sense RNA probes respectively. The RNA probes were digoxigenin-labeled and transcribed using T7 and Sp6 promoters. The Dig RNA labeling mix, T7 polymerase and SP6 polymerase were obtained from Roche Applied Sciences.

Sema6D insitu probe preparation:

The Chicken EST 225n10 was used to produce antisense and sense RNA probes. The Plasmid (pBSKS+ from Stratagene) was linearized using NotI and EcoRI respectively. The RNA probes were digoxigenin labeled and transcribed using T3 and T7 promoters. The Dig RNA labeling mix, T7 polymerase and SP6 polymerase were obtained from Roche Applied Sciences.

Procedure for in situ hybridization using cryostat sections:

Frozen embryos were cut on a cryostat (Leica) at 20µm mounted on SuperFrost Plus slides (Fischer scientific), fixed in 4% paraformaldehyde (Sigma) for 30 min, washed briefly with phosphate buffered saline (PBS), followed by washing in 2X SSC and then treated with 0.25% acetic anhydride in triethanolamine for 10 min. Prehybridization was carried out for 3 hours at 56^{o}C in prehybridization mix (50% formamide, 5X SSC, 5X Denhardt's, 250µg ml^{-1} yeast tRNA and 500µg ml^{-1} herring sperm DNA). Hybridization was performed overnight at 56^{o}C in prehybridization mix containing the sense and antisense probes, respectively. Washing was carried out at 56^{o}C with 5X SSC, 2X SSC, 0.2X SSC for 5 min each and 50% formamide /0.2X SSC for 20 min. followed by successive washing in 0.2X SSC and 1x TE (Tris-EDTA, pH 8) at RT for 5 min. Blocking was carried out with 3% milk powder in 1X TE and then incubated with Anti-Dig-AP-Fab (1:5000) (Roche). Immunological detection was carried out using nitro blue tetrazolium (NBT) and 5-bromo 3-chloro 4-indolyl phosphate

(BCIP) (Roche) as substrates in 1X AP buffer (100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, pH 9.5). Blue-purple colour was judged to be a positive signal. Sense controls were included in all experiments as controls for non-specific background signals.

Procedure for wholemount in situ hybridization:

For wholemount in situ hybridizations embryos were fixed in 4% PFA in 4 ℃ overnight. They were rinsed with PBT (PBS with 0.1% Triton-X-100) twice for 10 minutes. Cavities that would trap the probe were opened. Embryos were dehydrated in a graded methanol series diluted in PBT (25%, 50%, 75% methanol) and then twice in 100% methanol. The embryos were re-hydrated by washing in graded methanol series in PBT (75%, 50%, 25%), ending with PBT. Embryos were treated with 20 µg/ml proteinase K in PBT for 5-20 minutes at RT (5 minutes for stages 3-6, 10 min for stages 6-12, 20 min for stages 12-25). Embryos were washed with PBT for 5 min and then re-fixed with 4% paraformaldehyde in PBT for 20 minutes followed by rinsing in PBT. Prehybridization was carried out in prehybridization mix (as mentioned above with additional 0.1% triton-X-100) at 56℃ overnight. Embryos were incubated in hybridization mix at 56℃ for 2-3 days in order to facilitate penetration of probe.

Post hybridization washes were carried out in 2 x SSC, 0.1 % CHAPS, 0.2 x SSC, 0.1 % CHAPS three times for 20 min each at 56 ℃, followed by washing in KTBT (100 mM Tris-HCl, 150 mM NaCl, 1 % Triton-X-100, pH 7.5) for 10 min. Embryos were pre-blocked in 10 % FCS in KTBT for 2-3 hrs and then incubated with Anti-Dig-AP-Fab (1:2000) (Roche) overnight at 4, followed by thorough washing in KTBT. Colour development was carried out as mentioned above. The substrates were mixed in NTMT (100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton-X-100, pH 9.5) Periodically the reaction was monitored and when a strong signal was observed, the reaction was stopped by washing several times with KTBT and 1X TE (pH 8).

Immunohistochemistry (whole mount and 25μm thick sections):

Injected and control embryos were analyzed using either intact embryos or transverse sections of 25μ thickness. The following primary antibodies were used: monoclonal antibody RMO270 (1:1000) (Zymed Laboratories), rabbit anti-GFP (1:500) (Dianova), Islet-1 and 1E8 supernatants (generated from cell line obtained from DSHB) and rabbit serum raised against axonin-1 (1:1000) (Stoeckli and Landmesser, 1995).

Primary antibodies were detected using the following secondary antibodies: goat anti-mouse IgG-Cy3 (1:250) (Zymed Laboratories) and goat anti-rabbit- Alexa 488 (1:250) (Molecular Probes).

Tissue sections and embryos were permeabilized with Triton-X 100. Intact embryos were incubated with 1% Triton-X 100 in PBS for one hour before staining while the 25μm sections were permeabilized during blocking with 0.1% TritonX-100 in 10% FCS in PBS. Tissue sections and intact embryos were then treated with 20mM lysine in 0.1M sodium phosphate buffer (pH 7.4) to prevent unspecific binding of antibodies for an hour. Blocking was carried out in 10% FCS in PBS with 0.1% Triton X-100 for 2 hours. Primary antibody incubation was carried out overnight at 4^0C and incubations with appropriate secondary antibodies were carried out at room temperature for 2-5 hours. Slides were mounted using IMMU-MOUNT (Thermo Shandon, PA).

Clearance of tissue:

In order to analyze intact embryos either processed for wholemount neurofilament staining or whole-mount in situ hybridization, the tissue had to be cleared. This process was carried out by dehydrating the embryos in progressive concentrations of methanol (25%, 50%, 75% and 100%) for half an hour each with a repeated wash in 100% methanol to ensure efficient dehydration. Subsequently these embryos were transferred into glass vials and cleared in BBBA (Benzyl Benzoate: Benzyl Alcohol (2:1)). The embryos were gently swirled in the BBBA mix till they turned translucent and then analyzed using a binocular equipped with fluorescence optics (Olympus SZX12) and Analysis 5 software.

In ovo RNA interference:

Preparation of long doublestranded RNA (dsRNA):

Doublestranded RNA for targeting Sema6A, 6B and 6D was produced from the plasmids mentioned above. The plasmids were linearized using the same restriction enzymes which were used to prepare cRNA probes for in situ hybridization. Two µgs of linearized plasmid were mixed with dNTPs (Roche) to obtain a final concentration of 4mM; two µl T3 or T7 RNA polymerase (15U/ µl; Roche), 4 µl of 5X transcription buffer and 0.5 µl RNasin (30 U; Promega) in a total volume of 20 µl. After completion of transcription (37°C for 4 hours), DNAse I (Roche) was added and the RNA was extracted using acidic phenol-chloroform (25:24:1 vol/vol/vol phenol/chloroform/isoamyl alcohol) and subsequently with chloroform/isoamyl alcohol (24:1 vol/vol). Following precipitation with ethanol, the RNA was dissolved in 20 µl RNase-free PBS. Subsequently, equal amounts of sense and anti-sense RNA were mixed, heated to 95°C for 5 minutes, and double-stranded RNAs were allowed to anneal by gradual cooling of the reaction mixture from 95°C to room temperature.

DsRNA injection and electroporation:

In ovo RNA interference was performed according to the method described by Pekarik et al. 2003. For dsRNA injections, we used glass electrodes with a tip diameter of 5µm. In summary, 0.1-0.5µl phosphate-buffered saline (PBS), containing either a mixture of, Sema6A or 6B or 6D dsRNA (200-500 ng/µl) and YFP plasmid (under the control of β-actin promoter), or plasmids encoding YFP alone were injected into the central canal of the chicken spinal cord. Five pulses were delivered at 18V for a pulse length of 50ms using a BTX Electroporator (Model Electro Square Porator T820). Before and after electroporation, a few drops of sterile PBS were added to cool the embryo. Platinum electrodes (BTX, Genotronics) of 4 mm length with a distance of 4 mm between anode and cathode were used. The number of pulses and the voltage were chosen depending on the age of the embryo. After two to three days, embryos were sacrificed and processed for immunofluorescence analysis.

At the time of injection, the embryos were staged according to Hamburger and Hamilton, 1951. Injections of dsRNA for Sema6A and Sema6D were carried out at stages 14-16, while those for Sema6B were carried out at stages 17-19. The embryos were injected into the central canal of the spinal cord at the lumbosacral level. A reporter plasmid encoding YFP was co-injected with the dsRNA to ensure the efficiency of the injection and to trace the cells that have received the dsRNA. Embryos were injected at stages 14-16 for Sema6A and 6D to ensure that the cells that later on occupy areas of expression, received the dsRNA. The dissections were carried out at stages 25-27. Embryos were processed for wholemount immunohistochemistry, in situ hybridizations and immunohistochemistry on cryostat sections. Moreover, some embryos were also used for wholemount neurofilament staining. The slides were analyzed using an Olympus BX51 and Analysis 5 software.

Appendix:

- **DEPC treated water:** 1 ml of Diethyl pyrocarbonate (DEPC) to 1L of distilled water and stirred overnight. The water is then autoclaved

- **20X PBS**:

NaCl	160g/l
KCl	4g/l
$Na_2HPO_4 \times 2H_2O$	28,8g/l
KH_2PO_4	4g/l

Volume was made up to 1L distilled water
And adjusted to pH 7.4. 1ml DEPC was added and stirred well overnight
and then autoclaved

- **20X SSC**:

NaCl	175g/l
Tri-sodium citrate dehydrate	88,3g/l

Volume was made up to 1L and adjusted to pH 7.0.
1ml Diethyl pyrocarbonate (DEPC) was added and stirred well overnight and then autoclaved

- **0.2M Sodium phosphate buffer:**

  $NaH_2PO_4.H_2O$ 5.52g/l
  $Na_2HPO_4.2H_2O$ 28.48g/l
  Dissolved in 1 liter of double distilled water and adjusted to pH7.4

- **20mM Lysine in 0.1 M sodium phosphate buffer:**

  Dissolve 2.92 g of Lysine in 500ml of 0.2M Sodium phosphate buffer (pH7.4). Add 500 ml of double distilled water. Adjust the pH to 7.4 with 6M HCl

- **Paraformaldehyde**: <u>10% PFA</u>:

  10 g PFA dissolved in 100ml double distilled water. Add 100µl of 1M NaOH. Warm it with constant stirring @ 65°C in a water bath.

  <u>4% PFA</u>:

  40 ml of 10% PFA and make the volume to 100 ml with 1XPBS

- **PBT**: 1X PBS with 0.1% Triton X-100
- **KTBT**: 100 mM Tris-HCl, 150 mM NaCl, 1 % Triton-X-100, pH 7.5
- **NTMT**: 100 mM Tris-HCl, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Triton-X-100, pH 9.
- **Proteinase K**: 10 mg/ml stock solution in sterile

H_2O

- **Prehybridization solution**: 50% Formamide, 5X SSC, 5X Denhardt's, 250µg /ml yeast total RNA, 500µg/ml herring sperm DNA, 0.1% Triton X-100 Volume made up with DEPC-Water
- **50x Denhardt's solution:** 1 % Ficoll 1g
  1 % BSA 1g
  (Bovine Serum Albumin, non-acetylated)
  1 % PVP (polyvinylpyrrolidone) 1g
  Volume made up to 100ml with DEPC-Water. Heated to 50°C on a stirrer, filtered through 0.45µm filter, aliquots 10ml stored at -20°C
- **Herring sperm DNA (hsDNA):**
  Lyophilized DNA is dissolved at the concentration of 20 mg/ml in DEPC treated water, aliquots of 1ml at minus 20°C
- **Yeast total RNA (ytRNA):** Extracted RNA is dissolved at the concentration of 10 mg/ml in DEPC treated water, aliquots of 1ml at -20°
- **NBT stock solution**: 75 mg/ml of nitro blue tetrazolium (NBT) salt in 70% dimethyl formamide
- **BCIP stock solution**: 50 mg/ml of bromochloro indole phosphate (BCIP) in dimethyl formamide
- **Levamisole**: 24 mg/ ml of levamisole hydrochloride in NTMT
- **10X detection wash buffer**:1 M Tris-base 121.1g

1.5M NaCl 87.7g
Dissolved in 800ml water, adjusted to pH 7.5 with approximately 70 ml concentrated HCl and volume was adjusted with water to 1 litre and autoclaved

- **10X TE**: Tris-Base (100mM) 12.1 g/l
  EDTA (10mM, pH:8) 3.72 g/l
  Volume was adjusted with distilled water to 1 litre and autoclaved
- **Blocking buffer**: 3% milk powder in 1X detection wash buffer
- **10X AP buffer without $MgCl_2$:** 1 M Tris-base 121.1g
  1.5M NaCl 58.4 g
  Dissolved in 800ml water, adjusted to pH 9.5 with a few drops of concentrated HCl and volume was adjusted with water to 1 litre and autoclaved
- **1M $MgCl_2$:** $MgCl_2$ x 6 H_20 203.3g
  Volume was adjusted with water to 1 litre and autoclaved
- **1X AP buffer with $MgCl_2$:** 100 ml of 10X AP buffer without $MgCl_2$ and 50 ml of 1 M $MgCl_2$ were mixed and volume was made up to 1 litre with distilled water.
- **Development solution:**

Stock solution		Final solution
240 µg/ml	Levamisole 24 mg/ml	100µl
337.5 µg/ml	NBT 75 mg/ml	45µl
175 µg/ml	BCIP 50 mg/ml	35µl

Volume adjusted to 10 ml with 1X AP buffer

Chapter 5
References

- Adams, D. H., and Scott, S. A. (1998). Response of "naive" cutaneous and muscle afferents to potential targets in vitro. Dev Biol *203*, 210-220.
- Adams, M. D., and Sekelsky, J. J. (2002). From sequence to phenotype: reverse genetics in Drosophila melanogaster. Nat Rev Genet *3*, 189-198.
- Adams, R. H., Betz, H., and Puschel, A. W. (1996). A novel class of murine semaphorins with homology to thrombospondin is differentially expressed during early embryogenesis. Mech Dev *57*, 33-45.
- Ara, J., Bannerman, P., Shaheen, F., and Pleasure, D. E. (2005). Schwann cell-autonomous role of neuropilin-2. J Neurosci Res *79*, 468-475.
- Augsburger, A., Schuchardt, A., Hoskins, S., Dodd, J., and Butler, S. (1999). BMPs as mediators of roof plate repulsion of commissural neurons. Neuron *24*, 127-141.
- Bachelder, R. E., Lipscomb, E. A., Lin, X., Wendt, M. A., Chadborn, N. H., Eickholt, B. J., and Mercurio, A. M. (2003). Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. Cancer Res *63*, 5230-5233.
- Bagnard, D., Lohrum, M., Uziel, D., Puschel, A. W., and Bolz, J. (1998). Semaphorins act as attractive and repulsive guidance signals during the development of cortical projections. Development *125*, 5043-5053.
- Bagnard, D., Thomasset, N., Lohrum, M., Puschel, A. W., and Bolz, J. (2000). Spatial distributions of guidance molecules regulate chemorepulsion and chemoattraction of growth cones. J Neurosci *20*, 1030-1035.
- Barberis, D., Artigiani, S., Casazza, A., Corso, S., Giordano, S., Love, C. A., Jones, E. Y., Comoglio, P. M., and Tamagnone, L. (2004). Plexin signaling hampers integrin-based adhesion, leading to Rho-kinase independent cell rounding, and inhibiting lamellipodia extension and cell motility. Faseb J *18*, 592-594.
- Barresi, M. J., Hutson, L. D., Chien, C. B., and Karlstrom, R. O. (2005). Hedgehog regulated Slit expression determines commissure and glial cell position in the zebrafish forebrain. Development *132*, 3643-3656.
- Battye, R., Stevens, A., Perry, R. L., and Jacobs, J. R. (2001). Repellent signaling by Slit requires the leucine-rich repeats. J Neurosci *21*, 4290-4298.
- Beckmann, M. P., Cerretti, D. P., Baum, P., Vanden Bos, T., James, L., Farrah, T., Kozlosky, C., Hollingsworth, T., Shilling, H., Maraskovsky, E., and et al. (1994). Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors. Embo J *13*, 3757-3762.
- Bielenberg, D. R., Hida, Y., Shimizu, A., Kaipainen, A., Kreuter, M., Kim, C. C., and Klagsbrun, M. (2004). Semaphorin 3F, a chemorepulsant for endothelial cells,

induces a poorly vascularized, encapsulated, nonmetastatic tumor phenotype. J Clin Invest *114*, 1260-1271.

- Bourikas, D., Pekarik, V., Baeriswyl, T., Grunditz, A., Sadhu, R., Nardo, M., and Stoeckli, E. T. (2005). Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord. Nat Neurosci *8*, 297-304.
- Bourikas, D., and Stoeckli, E. T. (2003). New tools for gene manipulation in chicken embryos. Oligonucleotides *13*, 411-419.
- Bovolenta, P., and Dodd, J. (1990). Guidance of commissural growth cones at the floor plate in embryonic rat spinal cord. Development *109*, 435-447.
- Brambilla, R., Schnapp, A., Casagranda, F., Labrador, J. P., Bergemann, A. D., Flanagan, J. G., Pasquale, E. B., and Klein, R. (1995). Membrane-bound LERK2 ligand can signal through three different Eph-related receptor tyrosine kinases. Embo J *14*, 3116-3126.
- Brors, D., Bodmer, D., Pak, K., Aletsee, C., Schafers, M., Dazert, S., and Ryan, A. F. (2003). EphA4 provides repulsive signals to developing cochlear ganglion neurites mediated through ephrin-B2 and -B3. J Comp Neurol *462*, 90-100.
- Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M., and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. Cell *96*, 795-806.
- Brummendorf, T., Hubert, M., Treubert, U., Leuschner, R., Tarnok, A., and Rathjen, F. G. (1993). The axonal recognition molecule F11 is a multifunctional protein: specific domains mediate interactions with Ng-CAM and restrictin. Neuron *10*, 711-727.
- Brummendorf, T., Kenwrick, S., and Rathjen, F. G. (1998). Neural cell recognition molecule L1: from cell biology to human hereditary brain malformations. Curr Opin Neurobiol *8*, 87-97.
- Brummendorf, T., and Rathjen, F. G. (1993). Axonal glycoproteins with immunoglobulin- and fibronectin type III-related domains in vertebrates: structural features, binding activities, and signal transduction. J Neurochem *61*, 1207-1219.
- Brummendorf, T., and Rathjen, F. G. (1995). Cell adhesion molecules 1: immunoglobulin superfamily. Protein Profile *2*, 963-1108.
- Brummendorf, T., and Rathjen, F. G. (1996). Structure/function relationships of axon-associated adhesion receptors of the immunoglobulin superfamily. Curr Opin Neurobiol *6*, 584-593.
- Buchman, V. L., and Davies, A. M. (1993). Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. Development *118*, 989-1001.
- Burden-Gulley, S. M., Payne, H. R., and Lemmon, V. (1995). Growth cones are actively influenced by substrate-bound adhesion molecules. J Neurosci *15*, 4370-4381.

- Burkhardt, C., Muller, M., Badde, A., Garner, C. C., Gundelfinger, E. D., and Puschel, A. W. (2005). Semaphorin 4B interacts with the post-synaptic density protein PSD-95/SAP90 and is recruited to synapses through a C-terminal PDZ-binding motif. FEBS Lett *579*, 3821-3828.
- Burstyn-Cohen, T., Tzarfaty, V., Frumkin, A., Feinstein, Y., Stoeckli, E., and Klar, A. (1999). F-Spondin is required for accurate pathfinding of commissural axons at the floor plate. Neuron *23*, 233-246.
- Cai, H., and Reed, R. R. (1999). Cloning and characterization of neuropilin-1-interacting protein: a PSD-95/Dlg/ZO-1 domain-containing protein that interacts with the cytoplasmic domain of neuropilin-1. J Neurosci *19*, 6519-6527.
- Campbell, D. S., and Holt, C. E. (2001). Chemotropic responses of retinal growth cones mediated by rapid local protein synthesis and degradation. Neuron *32*, 1013-1026.
- Chan, S. S., Zheng, H., Su, M. W., Wilk, R., Killeen, M. T., Hedgecock, E. M., and Culotti, J. G. (1996). UNC-40, a C. elegans homolog of DCC (Deleted in Colorectal Cancer), is required in motile cells responding to UNC-6 netrin cues. Cell *87*, 187-195.
- Chedotal, A., Del Rio, J. A., Ruiz, M., He, Z., Borrell, V., de Castro, F., Ezan, F., Goodman, C. S., Tessier-Lavigne, M., Sotelo, C., and Soriano, E. (1998). Semaphorins III and IV repel hippocampal axons via two distinct receptors. Development *125*, 4313-4323.
- Chen, H., Bagri, A., Zupicich, J. A., Zou, Y., Stoeckli, E., Pleasure, S. J., Lowenstein, D. H., Skarnes, W. C., Chedotal, A., and Tessier-Lavigne, M. (2000). Neuropilin-2 regulates the development of selective cranial and sensory nerves and hippocampal mossy fiber projections. Neuron *25*, 43-56.
- Chen, H., Chedotal, A., He, Z., Goodman, C. S., and Tessier-Lavigne, M. (1997). Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. Neuron *19*, 547-559.
- Cheng, H. J., Bagri, A., Yaron, A., Stein, E., Pleasure, S. J., and Tessier-Lavigne, M. (2001). Plexin-A3 mediates semaphorin signaling and regulates the development of hippocampal axonal projections. Neuron *32*, 249-263.
- Cheng, H. J., and Flanagan, J. G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. Cell *79*, 157-168.
- Cheng, H. J., Nakamoto, M., Bergemann, A. D., and Flanagan, J. G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell *82*, 371-381.
- Chilton, J. K., and Guthrie, S. (2003). Cranial expression of class 3 secreted semaphorins and their neuropilin receptors. Dev Dyn *228*, 726-733.

- Chu-Wang, I. W., and Oppenheim, R. W. (1978). Cell death of motoneurons in the chick embryo spinal cord. II. A quantitative and qualitative analysis of degeneration in the ventral root, including evidence for axon outgrowth and limb innervation prior to cell death. J Comp Neurol *177*, 59-85.
- Cloutier, J. F., Sahay, A., Chang, E. C., Tessier-Lavigne, M., Dulac, C., Kolodkin, A. L., and Ginty, D. D. (2004). Differential requirements for semaphorin 3F and Slit-1 in axonal targeting, fasciculation, and segregation of olfactory sensory neuron projections. J Neurosci *24*, 9087-9096.
- Cohen, S., Funkelstein, L., Livet, J., Rougon, G., Henderson, C. E., Castellani, V., and Mann, F. (2005). A semaphorin code defines subpopulations of spinal motor neurons during mouse development. Eur J Neurosci *21*, 1767-1776.
- Colamarino, S. A., and Tessier-Lavigne, M. (1995a). The axonal chemoattractant netrin-1 is also a chemorepellent for trochlear motor axons. Cell *81*, 621-629.
- Colamarino, S. A., and Tessier-Lavigne, M. (1995b). The role of the floor plate in axon guidance. Annu Rev Neurosci *18*, 497-529.
- Collet, P., Domenjoud, L., Devignes, M. D., Murad, H., Schohn, H., and Dauca, M. (2004). The human semaphorin 6B gene is down regulated by PPARs. Genomics *83*, 1141-1150.
- Comeau, M. R., Johnson, R., DuBose, R. F., Petersen, M., Gearing, P., VandenBos, T., Park, L., Farrah, T., Buller, R. M., Cohen, J. I.*, et al.* (1998). A poxvirus-encoded semaphorin induces cytokine production from monocytes and binds to a novel cellular semaphorin receptor, VESPR. Immunity *8*, 473-482.
- Conrotto, P., Corso, S., Gamberini, S., Comoglio, P. M., and Giordano, S. (2004). Interplay between scatter factor receptors and B plexins controls invasive growth. Oncogene *23*, 5131-5137.
- Conrotto, P., Valdembri, D., Corso, S., Serini, G., Tamagnone, L., Comoglio, P. M., Bussolino, F., and Giordano, S. (2005). Sema4D induces angiogenesis through Met recruitment by Plexin B1. Blood *105*, 4321-4329.
- Cooper, H. M. (2002). Axon guidance receptors direct growth cone pathfinding: rivalry at the leading edge. Int J Dev Biol *46*, 621-631.
- Cooper, H. M., Gad, J. M., and Keeling, S. L. (1999). The Deleted in Colorectal Cancer netrin guidance system: a molecular strategy for neuronal navigation. Clin Exp Pharmacol Physiol *26*, 749-751.
- Correa, R. G., Sasahara, R. M., Bengtson, M. H., Katayama, M. L., Salim, A. C., Brentani, M. M., Sogayar, M. C., de Souza, S. J., and Simpson, A. J. (2001). Human semaphorin 6B [(HSA)SEMA6B], a novel human class 6 semaphorin gene: alternative splicing and all-trans-retinoic acid-dependent downregulation in glioblastoma cell lines. Genomics *73*, 343-348.
- D'Amico-Martel, A., and Noden, D. M. (1983). Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. Am J Anat *166*, 445-468.

- Davis, B. M., Frank, E., Johnson, F. A., and Scott, S. A. (1989). Development of central projections of lumbosacral sensory neurons in the chick. J Comp Neurol *279*, 556-566.
- Davy, A., and Soriano, P. (2005). Ephrin signaling in vivo: look both ways. Dev Dyn *232*, 1-10.
- de Castro, F., Hu, L., Drabkin, H., Sotelo, C., and Chedotal, A. (1999). Chemoattraction and chemorepulsion of olfactory bulb axons by different secreted semaphorins. J Neurosci *19*, 4428-4436.
- de Winter, F., Cui, Q., Symons, N., Verhaagen, J., and Harvey, A. R. (2004). Expression of class-3 semaphorins and their receptors in the neonatal and adult rat retina. Invest Ophthalmol Vis Sci *45*, 4554-4562.
- Dhanabal, M., Wu, F., Alvarez, E., McQueeney, K. D., Jeffers, M., Macdougall, J., Boldog, F. L., Hackett, C., Shenoy, S., Khramtsov, N.*, et al.* (2005). Recombinant Semaphorin 6A-1 Ectodomain Inhibits In Vivo Growth Factor and Tumor Cell Line-Induced Angiogenesis. Cancer Biol Ther *4*, 659-668.
- Dou, C., Ye, X., Stewart, C., Lai, E., and Li, S. C. (1997). TWH regulates the development of subsets of spinal cord neurons. Neuron *18*, 539-551.
- Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., and Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell *82*, 359-370.
- Eberhart, J., Barr, J., O'Connell, S., Flagg, A., Swartz, M. E., Cramer, K. S., Tosney, K. W., Pasquale, E. B., and Krull, C. E. (2004). Ephrin-A5 exerts positive or inhibitory effects on distinct subsets of EphA4-positive motor neurons. J Neurosci *24*, 1070-1078.
- Eberhart, J., Swartz, M., Koblar, S. A., Pasquale, E. B., Tanaka, H., and Krull, C. E. (2000). Expression of EphA4, ephrin-A2 and ephrin-A5 during axon outgrowth to the hindlimb indicates potential roles in pathfinding. Dev Neurosci *22*, 237-250.
- Eberhart, J., Swartz, M. E., Koblar, S. A., Pasquale, E. B., and Krull, C. E. (2002). EphA4 constitutes a population-specific guidance cue for motor neurons. Dev Biol *247*, 89-101.
- Eckhardt, F., Behar, O., Calautti, E., Yonezawa, K., Nishimoto, I., and Fishman, M. C. (1997). A novel transmembrane semaphorin can bind c-src. Mol Cell Neurosci *9*, 409-419.
- Edlund, T., and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. Cell *96*, 211-224.
- Eickholt, B. J., Mackenzie, S. L., Graham, A., Walsh, F. S., and Doherty, P. (1999). Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. Development *126*, 2181-2189.

- Eide, A. L., Glover, J., Kjaerulff, O., and Kiehn, O. (1999). Characterization of commissural interneurons in the lumbar region of the neonatal rat spinal cord. J Comp Neurol *403*, 332-345.
- Encinas, J. A., Kikuchi, K., Chedotal, A., de Castro, F., Goodman, C. S., and Kimura, T. (1999). Cloning, expression, and genetic mapping of Sema W, a member of the semaphorin family. Proc Natl Acad Sci U S A *96*, 2491-2496.
- Ericson, J., Thor, S., Edlund, T., Jessell, T. M., and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. Science *256*, 1555-1560.
- Farinas, I., Cano-Jaimez, M., Bellmunt, E., and Soriano, M. (2002). Regulation of neurogenesis by neurotrophins in developing spinal sensory ganglia. Brain Res Bull *57*, 809-816.
- Fazeli, A., Dickinson, S. L., Hermiston, M. L., Tighe, R. V., Steen, R. G., Small, C. G., Stoeckli, E. T., Keino-Masu, K., Masu, M., Rayburn, H.*, et al.* (1997). Phenotype of mice lacking functional Deleted in colorectal cancer (Dcc) gene. Nature *386*, 796-804.
- Feiner, L., Webber, A. L., Brown, C. B., Lu, M. M., Jia, L., Feinstein, P., Mombaerts, P., Epstein, J. A., and Raper, J. A. (2001). Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. Development *128*, 3061-3070.
- Feng, G., Laskowski, M. B., Feldheim, D. A., Wang, H., Lewis, R., Frisen, J., Flanagan, J. G., and Sanes, J. R. (2000). Roles for ephrins in positionally selective synaptogenesis between motor neurons and muscle fibers. Neuron *25*, 295-306.
- Fiore, R., and Puschel, A. W. (2003). The function of semaphorins during nervous system development. Front Biosci *8*, s484-499.
- Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.
- Fitzli, D., Stoeckli, E. T., Kunz, S., Siribour, K., Rader, C., Kunz, B., Kozlov, S. V., Buchstaller, A., Lane, R. P., Suter, D. M.*, et al.* (2000). A direct interaction of axonin-1 with NgCAM-related cell adhesion molecule (NrCAM) results in guidance, but not growth of commissural axons. J Cell Biol *149*, 951-968.
- Flanagan, J. G., and Vanderhaeghen, P. (1998). The ephrins and Eph receptors in neural development. Annu Rev Neurosci *21*, 309-345.
- Fouvet, B. (1973). [Innervation and morphogenesis of the leg in the chick embryo. I. Development of normal innervation]. Arch Anat Microsc Morphol Exp *62*, 269-280.
- Fraher, J. (2002). Axons and glial interfaces: ultrastructural studies. J Anat *200*, 415-430.
- Fraher, J. P. (1997). Axon-glial relationships in early CNS-PNS transitional zone development: an ultrastructural study. J Neurocytol *26*, 41-52.

- Fujisawa, H., Kitsukawa, T., Kawakami, A., Takagi, S., Shimizu, M., and Hirata, T. (1997). Roles of a neuronal cell-surface molecule, neuropilin, in nerve fiber fasciculation and guidance. Cell Tissue Res *290*, 465-470.
- Gavazzi, I., Stonehouse, J., Sandvig, A., Reza, J. N., Appiah-Kubi, L. S., Keynes, R., and Cohen, J. (2000). Peripheral, but not central, axotomy induces neuropilin-1 mRNA expression in adult large diameter primary sensory neurons. J Comp Neurol *423*, 492-499.
- Gherardi, E., Love, C. A., Esnouf, R. M., and Jones, E. Y. (2004). The sema domain. Curr Opin Struct Biol *14*, 669-678.
- Giger, R. J., Cloutier, J. F., Sahay, A., Prinjha, R. K., Levengood, D. V., Moore, S. E., Pickering, S., Simmons, D., Rastan, S., Walsh, F. S.*, et al.* (2000). Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. Neuron *25*, 29-41.
- Giger, R. J., Urquhart, E. R., Gillespie, S. K., Levengood, D. V., Ginty, D. D., and Kolodkin, A. L. (1998). Neuropilin-2 is a receptor for semaphorin IV: insight into the structural basis of receptor function and specificity. Neuron *21*, 1079-1092.
- Giger, R. J., Wolfer, D. P., De Wit, G. M., and Verhaagen, J. (1996). Anatomy of rat semaphorin III/collapsin-1 mRNA expression and relationship to developing nerve tracts during neuroembryogenesis. J Comp Neurol *375*, 378-392.
- Giordano, S., Corso, S., Conrotto, P., Artigiani, S., Gilestro, G., Barberis, D., Tamagnone, L., and Comoglio, P. M. (2002). The semaphorin 4D receptor controls invasive growth by coupling with Met. Nat Cell Biol *4*, 720-724.
- Gitler, A. D., Lu, M. M., and Epstein, J. A. (2004). PlexinD1 and semaphorin signaling are required in endothelial cells for cardiovascular development. Dev Cell *7*, 107-116.
- Goldberg, J. L., Vargas, M. E., Wang, J. T., Mandemakers, W., Oster, S. F., Sretavan, D. W., and Barres, B. A. (2004). An oligodendrocyte lineage-specific semaphorin, Sema5A, inhibits axon growth by retinal ganglion cells. J Neurosci *24*, 4989-4999.
- Guan, W., and Condic, M. L. (2003). Characterization of Netrin-1, Neogenin and cUNC-5H3 expression during chick dorsal root ganglia development. Gene Expr Patterns *3*, 369-373.
- Guthrie, S., and Pini, A. (1995). Chemorepulsion of developing motor axons by the floor plate. Neuron *14*, 1117-1130.
- Hall, D. H., Winfrey, V. P., Blaeuer, G., Hoffman, L. H., Furuta, T., Rose, K. L., Hobert, O., and Greenstein, D. (1999). Ultrastructural features of the adult hermaphrodite gonad of Caenorhabditis elegans: relations between the germ line and soma. Dev Biol *212*, 101-123.
- Hall, K. T., Boumsell, L., Schultze, J. L., Boussiotis, V. A., Dorfman, D. M., Cardoso, A. A., Bensussan, A., Nadler, L. M., and Freeman, G. J. (1996). Human CD100, a

novel leukocyte semaphorin that promotes B-cell aggregation and differentiation. Proc Natl Acad Sci U S A *93*, 11780-11785.

- Hamburger, V. (1958). Regression versus peripheral control of differentiation in motor hypoplasia. Am J Anat *102*, 365-409.
- Hamburger, V. (1975). Cell death in the development of the lateral motor column of the chick embryo. J Comp Neurol *160*, 535-546.
- Hamburger, V. (1977). The developmental history of the motor neuron. Neurosci Res Program Bull *15 Suppl*, iii-37.
- Hannon, G. J. (2002). RNA interference. Nature *418*, 244-251.
- Harris, R., Sabatelli, L. M., and Seeger, M. A. (1996). Guidance cues at the Drosophila CNS midline: identification and characterization of two Drosophila Netrin/UNC-6 homologs. Neuron *17*, 217-228.
- He, Z., and Tessier-Lavigne, M. (1997). Neuropilin is a receptor for the axonal chemorepellent Semaphorin III. Cell *90*, 739-751.
- Hindges, R., McLaughlin, T., Genoud, N., Henkemeyer, M., and O'Leary, D. D. (2002). EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. Neuron *35*, 475-487.
- Holder, N., and Klein, R. (1999). Eph receptors and ephrins: effectors of morphogenesis. Development *126*, 2033-2044.
- Holmes, S., Downs, A. M., Fosberry, A., Hayes, P. D., Michalovich, D., Murdoch, P., Moores, K., Fox, J., Deen, K., Pettman, G.*, et al.* (2002). Sema7A is a potent monocyte stimulator. Scand J Immunol *56*, 270-275.
- Honig, M. G. (1982). The development of sensory projection patterns in embryonic chick hind limb. J Physiol *330*, 175-202.
- Honig, M. G., Lance-Jones, C., and Landmesser, L. (1986). The development of sensory projection patterns in embryonic chick hindlimb under experimental conditions. Dev Biol *118*, 532-548.
- Huber, A. B., Kolodkin, A. L., Ginty, D. D., and Cloutier, J. F. (2003). Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. Annu Rev Neurosci *26*, 509-563.
- Imondi, R., and Kaprielian, Z. (2001). Commissural axon pathfinding on the contralateral side of the floor plate: a role for B-class ephrins in specifying the dorsoventral position of longitudinally projecting commissural axons. Development *128*, 4859-4871.
- Inagaki, S., Ohoka, Y., Sugimoto, H., Fujioka, S., Amazaki, M., Kurinami, H., Miyazaki, N., Tohyama, M., and Furuyama, T. (2001). Sema4c, a transmembrane semaphorin, interacts with a post-synaptic density protein, PSD-95. J Biol Chem *276*, 9174-9181.

- Ishii, N., Wadsworth, W. G., Stern, B. D., Culotti, J. G., and Hedgecock, E. M. (1992). UNC-6, a laminin-related protein, guides cell and pioneer axon migrations in C. elegans. Neuron *9*, 873-881.
- Itasaki, N., Bel-Vialar, S., and Krumlauf, R. (1999). 'Shocking' developments in chick embryology: electroporation and in ovo gene expression. Nat Cell Biol *1*, E203-207.
- Jackson, I. J. (2001a). Mouse genomics: making sense of the sequence. Curr Biol *11*, R311-314.
- Jackson, I. J. (2001b). Mouse mutagenesis on target. Nat Genet *28*, 198-200.
- Jacob, J., Hacker, A., and Guthrie, S. (2001). Mechanisms and molecules in motor neuron specification and axon pathfinding. Bioessays *23*, 582-595.
- Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet *1*, 20-29.
- Jones, L., Lopez-Bendito, G., Gruss, P., Stoykova, A., and Molnar, Z. (2002). Pax6 is required for the normal development of the forebrain axonal connections. Development *129*, 5041-5052.
- Kameyama, T., Murakami, Y., Suto, F., Kawakami, A., Takagi, S., Hirata, T., and Fujisawa, H. (1996a). Identification of a neuronal cell surface molecule, plexin, in mice. Biochem Biophys Res Commun *226*, 524-529.
- Kameyama, T., Murakami, Y., Suto, F., Kawakami, A., Takagi, S., Hirata, T., and Fujisawa, H. (1996b). Identification of plexin family molecules in mice. Biochem Biophys Res Commun *226*, 396-402.
- Kania, A., Johnson, R. L., and Jessell, T. M. (2000). Coordinate roles for LIM homeobox genes in directing the dorsoventral trajectory of motor axons in the vertebrate limb. Cell *102*, 161-173.
- Kaprielian, Z., Runko, E., and Imondi, R. (2001). Axon guidance at the midline choice point. Dev Dyn *221*, 154-181.
- Kawakami, A., Kitsukawa, T., Takagi, S., and Fujisawa, H. (1996). Developmentally regulated expression of a cell surface protein, neuropilin, in the mouse nervous system. J Neurobiol *29*, 1-17.
- Kawasaki, T., Bekku, Y., Suto, F., Kitsukawa, T., Taniguchi, M., Nagatsu, I., Nagatsu, T., Itoh, K., Yagi, T., and Fujisawa, H. (2002). Requirement of neuropilin 1-mediated Sema3A signals in patterning of the sympathetic nervous system. Development *129*, 671-680.
- Kawasaki, T., Kitsukawa, T., Bekku, Y., Matsuda, Y., Sanbo, M., Yagi, T., and Fujisawa, H. (1999). A requirement for neuropilin-1 in embryonic vessel formation. Development *126*, 4895-4902.
- Keino-Masu, K., Masu, M., Hinck, L., Leonardo, E. D., Chan, S. S., Culotti, J. G., and Tessier-Lavigne, M. (1996). Deleted in Colorectal Cancer (DCC) encodes a netrin receptor. Cell *87*, 175-185.

- Kennedy, T. E., Serafini, T., de la Torre, J. R., and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425-435.
- Kerjan, G., Dolan, J., Haumaitre, C., Schneider-Maunoury, S., Fujisawa, H., Mitchell, K. J., and Chedotal, A. (2005). The transmembrane semaphorin Sema6A controls cerebellar granule cell migration. Nat Neurosci.
- Keynes, R., Tannahill, D., Morgenstern, D. A., Johnson, A. R., Cook, G. M., and Pini, A. (1997). Surround repulsion of spinal sensory axons in higher vertebrate embryos. Neuron *18*, 889-897.
- Keynes, R. J., and Stern, C. D. (1984). Segmentation in the vertebrate nervous system. Nature *310*, 786-789.
- Kidd, T., Bland, K. S., and Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. Cell *96*, 785-794.
- Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M., Goodman, C. S., and Tear, G. (1998a). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. Cell *92*, 205-215.
- Kidd, T., Russell, C., Goodman, C. S., and Tear, G. (1998b). Dosage-sensitive and complementary functions of roundabout and commissureless control axon crossing of the CNS midline. Neuron *20*, 25-33.
- Kikuchi, K., Chedotal, A., Hanafusa, H., Ujimasa, Y., de Castro, F., Goodman, C. S., and Kimura, T. (1999). Cloning and characterization of a novel class VI semaphorin, semaphorin Y. Mol Cell Neurosci *13*, 9-23.
- Kikuchi, K., Ishida, H., and Kimura, T. (1997). Molecular cloning of a novel member of semaphorin family genes, semaphorin Z. Brain Res Mol Brain Res *51*, 229-237.
- Kitsukawa, T., Shimizu, M., Sanbo, M., Hirata, T., Taniguchi, M., Bekku, Y., Yagi, T., and Fujisawa, H. (1997). Neuropilin-semaphorin III/D-mediated chemorepulsive signals play a crucial role in peripheral nerve projection in mice. Neuron *19*, 995-1005.
- Klein, R. (2001). Excitatory Eph receptors and adhesive ephrin ligands. Curr Opin Cell Biol *13*, 196-203.
- Klostermann, A., Lutz, B., Gertler, F., and Behl, C. (2000). The orthologous human and murine semaphorin 6A-1 proteins (SEMA6A-1/Sema6A-1) bind to the enabled/vasodilator-stimulated phosphoprotein-like protein (EVL) via a novel carboxyl-terminal zyxin-like domain. J Biol Chem *275*, 39647-39653.
- Knoll, B., Zarbalis, K., Wurst, W., and Drescher, U. (2001). A role for the EphA family in the topographic targeting of vomeronasal axons. Development *128*, 895-906.
- Kobayashi, H., Koppel, A. M., Luo, Y., and Raper, J. A. (1997). A role for collapsin-1 in olfactory and cranial sensory axon guidance. J Neurosci *17*, 8339-8352.

- Kolodkin, A. L., and Ginty, D. D. (1997). Steering clear of semaphorins: neuropilins sound the retreat. Neuron *19*, 1159-1162.
- Kolodkin, A. L., Levengood, D. V., Rowe, E. G., Tai, Y. T., Giger, R. J., and Ginty, D. D. (1997). Neuropilin is a semaphorin III receptor. Cell *90*, 753-762.
- Kolodkin, A. L., Matthes, D. J., and Goodman, C. S. (1993). The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. Cell *75*, 1389-1399.
- Kolodkin, A. L., Matthes, D. J., O'Connor, T. P., Patel, N. H., Admon, A., Bentley, D., and Goodman, C. S. (1992). Fasciclin IV: sequence, expression, and function during growth cone guidance in the grasshopper embryo. Neuron *9*, 831-845.
- Kolodziej, P. A., Timpe, L. C., Mitchell, K. J., Fried, S. R., Goodman, C. S., Jan, L. Y., and Jan, Y. N. (1996). frazzled encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. Cell *87*, 197-204.
- Koppel, A. M., Feiner, L., Kobayashi, H., and Raper, J. A. (1997). A 70 amino acid region within the semaphorin domain activates specific cellular response of semaphorin family members. Neuron *19*, 531-537.
- Kozlosky, C. J., Maraskovsky, E., McGrew, J. T., VandenBos, T., Teepe, M., Lyman, S. D., Srinivasan, S., Fletcher, F. A., Gayle, R. B., 3rd, Cerretti, D. P., and et al. (1995). Ligands for the receptor tyrosine kinases hek and elk: isolation of cDNAs encoding a family of proteins. Oncogene *10*, 299-306.
- Krull, C. E., and Koblar, S. A. (2000). Motor axon pathfinding in the peripheral nervous system. Brain Res Bull *53*, 479-487.
- Kullander, K., Butt, S. J., Lebret, J. M., Lundfald, L., Restrepo, C. E., Rydstrom, A., Klein, R., and Kiehn, O. (2003). Role of EphA4 and EphrinB3 in local neuronal circuits that control walking. Science *299*, 1889-1892.
- Kullander, K., Croll, S. D., Zimmer, M., Pan, L., McClain, J., Hughes, V., Zabski, S., DeChiara, T. M., Klein, R., Yancopoulos, G. D., and Gale, N. W. (2001a). Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. Genes Dev *15*, 877-888.
- Kullander, K., Mather, N. K., Diella, F., Dottori, M., Boyd, A. W., and Klein, R. (2001b). Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron *29*, 73-84.
- Kumanogoh, A., and Kikutani, H. (2003a). Immune semaphorins: a new area of semaphorin research. J Cell Sci *116*, 3463-3470.
- Kumanogoh, A., and Kikutani, H. (2003b). Roles of the semaphorin family in immune regulation. Adv Immunol *81*, 173-198.
- Kumanogoh, A., Marukawa, S., Suzuki, K., Takegahara, N., Watanabe, C., Ch'ng, E., Ishida, I., Fujimura, H., Sakoda, S., Yoshida, K., and Kikutani, H. (2002). Class IV

semaphorin Sema4A enhances T-cell activation and interacts with Tim-2. Nature *419*, 629-633.

- Kumanogoh, A., Shikina, T., Watanabe, C., Takegahara, N., Suzuki, K., Yamamoto, M., Takamatsu, H., Prasad, D. V., Mizui, M., Toyofuku, T.*, et al.* (2005). Requirement for CD100-CD72 interactions in fine-tuning of B-cell antigen receptor signaling and homeostatic maintenance of the B-cell compartment. Int Immunol *17*, 1277-1282.
- Kumanogoh, A., Watanabe, C., Lee, I., Wang, X., Shi, W., Araki, H., Hirata, H., Iwahori, K., Uchida, J., Yasui, T.*, et al.* (2000). Identification of CD72 as a lymphocyte receptor for the class IV semaphorin CD100: a novel mechanism for regulating B cell signaling. Immunity *13*, 621-631.
- Kury, P., Abankwa, D., Kruse, F., Greiner-Petter, R., and Muller, H. W. (2004). Gene expression profiling reveals multiple novel intrinsic and extrinsic factors associated with axonal regeneration failure. Eur J Neurosci *19*, 32-42.
- Lallier, T. E. (2004). Semaphorin profiling of periodontal fibroblasts and osteoblasts. J Dent Res *83*, 677-682.
- Lance-Jones, C. (1988). Development of neuromuscular connections: guidance of motoneuron axons to muscles in the embryonic chick hindlimb. Ciba Found Symp *138*, 97-115.
- Lance-Jones, C., and Landmesser, L. (1980). Motoneurone projection patterns in embryonic chick limbs following partial deletions of the spinal cord. J Physiol *302*, 559-580.
- Lance-Jones, C., and Landmesser, L. (1981). Pathway selection by chick lumbosacral motoneurons during normal development. Proc R Soc Lond B Biol Sci *214*, 1-18.
- Landmesser, L. (1978a). The development of motor projection patterns in the chick hind limb. J Physiol *284*, 391-414.
- Landmesser, L. (1978b). The distribution of motoneurones supplying chick hind limb muscles. J Physiol *284*, 371-389.
- Landmesser, L., Dahm, L., Schultz, K., and Rutishauser, U. (1988). Distinct roles for adhesion molecules during innervation of embryonic chick muscle. Dev Biol *130*, 645-670.
- Landmesser, L., Dahm, L., Tang, J. C., and Rutishauser, U. (1990). Polysialic acid as a regulator of intramuscular nerve branching during embryonic development. Neuron *4*, 655-667.
- Landmesser, L., and Honig, M. G. (1986). Altered sensory projections in the chick hind limb following the early removal of motoneurons. Dev Biol *118*, 511-531.
- Landmesser, L., and Morris, D. G. (1975). The development of functional innervation in the hind limb of the chick embryo. J Physiol *249*, 301-326.

- Lange, C., Liehr, T., Goen, M., Gebhart, E., Fleckenstein, B., and Ensser, A. (1998). New eukaryotic semaphorins with close homology to semaphorins of DNA viruses. Genomics *51*, 340-350.
- Laskowski, M. B., and Sanes, J. R. (1987). Topographic mapping of motor pools onto skeletal muscles. J Neurosci *7*, 252-260.
- Laskowski, M. B., and Sanes, J. R. (1988). Topographically selective reinnervation of adult mammalian skeletal muscles. J Neurosci *8*, 3094-3099.
- Lee, S. K., and Pfaff, S. L. (2001). Transcriptional networks regulating neuronal identity in the developing spinal cord. Nat Neurosci *4 Suppl*, 1183-1191.
- Leighton, P. A., Mitchell, K. J., Goodrich, L. V., Lu, X., Pinson, K., Scherz, P., Skarnes, W. C., and Tessier-Lavigne, M. (2001). Defining brain wiring patterns and mechanisms through gene trapping in mice. Nature *410*, 174-179.
- Leonardo, E. D., Hinck, L., Masu, M., Keino-Masu, K., Fazeli, A., Stoeckli, E. T., Ackerman, S. L., Weinberg, R. A., and Tessier-Lavigne, M. (1997). Guidance of developing axons by netrin-1 and its receptors. Cold Spring Harb Symp Quant Biol *62*, 467-478.
- Li, H. S., Chen, J. H., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z. H., Nash, W., Gick, C.*, et al.* (1999). Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. Cell *96*, 807-818.
- Lin, J. H., Saito, T., Anderson, D. J., Lance-Jones, C., Jessell, T. M., and Arber, S. (1998). Functionally related motor neuron pool and muscle sensory afferent subtypes defined by coordinate ETS gene expression. Cell *95*, 393-407.
- Lindsay, R. M., and Rohrer, H. (1985). Placodal sensory neurons in culture: nodose ganglion neurons are unresponsive to NGF, lack NGF receptors but are supported by a liver-derived neurotrophic factor. Dev Biol *112*, 30-48.
- Liu, J. P., Laufer, E., and Jessell, T. M. (2001). Assigning the positional identity of spinal motor neurons: rostrocaudal patterning of Hox-c expression by FGFs, Gdf11, and retinoids. Neuron *32*, 997-1012.
- Liu, Y., and Halloran, M. C. (2005). Central and peripheral axon branches from one neuron are guided differentially by Semaphorin3D and transient axonal glycoprotein-1. J Neurosci *25*, 10556-10563.
- Livesey, F. J. (1999). Netrins and netrin receptors. Cell Mol Life Sci *56*, 62-68.
- Livesey, F. J., and Hunt, S. P. (1997). Netrin and netrin receptor expression in the embryonic mammalian nervous system suggests roles in retinal, striatal, nigral, and cerebellar development. Mol Cell Neurosci *8*, 417-429.
- Long, H., Sabatier, C., Ma, L., Plump, A., Yuan, W., Ornitz, D. M., Tamada, A., Murakami, F., Goodman, C. S., and Tessier-Lavigne, M. (2004). Conserved roles for Slit and Robo proteins in midline commissural axon guidance. Neuron *42*, 213-223.

- Lu, Q., Sun, E. E., and Flanagan, J. G. (2004). Analysis of PDZ-RGS3 function in ephrin-B reverse signaling. Methods Enzymol *390*, 120-128.
- Lu, Q., Sun, E. E., Klein, R. S., and Flanagan, J. G. (2001). Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. Cell *105*, 69-79.
- Luo, Y., Raible, D., and Raper, J. A. (1993). Collapsin: a protein in brain that induces the collapse and paralysis of neuronal growth cones. Cell *75*, 217-227.
- Luo, Y., Shepherd, I., Li, J., Renzi, M. J., Chang, S., and Raper, J. A. (1995). A family of molecules related to collapsin in the embryonic chick nervous system. Neuron *14*, 1131-1140.
- Lyuksyutova, A. I., Lu, C. C., Milanesio, N., King, L. A., Guo, N., Wang, Y., Nathans, J., Tessier-Lavigne, M., and Zou, Y. (2003). Anterior-posterior guidance of commissural axons by Wnt-frizzled signaling. Science *302*, 1984-1988.
- Mann, F., Ray, S., Harris, W., and Holt, C. (2002). Topographic mapping in dorsoventral axis of the Xenopus retinotectal system depends on signaling through ephrin-B ligands. Neuron *35*, 461-473.
- Maro, G. S., Vermeren, M., Voiculescu, O., Melton, L., Cohen, J., Charnay, P., and Topilko, P. (2004). Neural crest boundary cap cells constitute a source of neuronal and glial cells of the PNS. Nat Neurosci *7*, 930-938.
- Masuda, K., Furuyama, T., Takahara, M., Fujioka, S., Kurinami, H., and Inagaki, S. (2004a). Sema4D stimulates axonal outgrowth of embryonic DRG sensory neurones. Genes Cells *9*, 821-829.
- Masuda, T., Fukamauchi, F., Takeda, Y., Fujisawa, H., Watanabe, K., Okado, N., and Shiga, T. (2004b). Developmental regulation of notochord-derived repulsion for dorsal root ganglion axons. Mol Cell Neurosci *25*, 217-227.
- Matthes, D. J., Sink, H., Kolodkin, A. L., and Goodman, C. S. (1995). Semaphorin II can function as a selective inhibitor of specific synaptic arborizations. Cell *81*, 631-639.
- Maurin, J. C., Delorme, G., Machuca-Gayet, I., Couble, M. L., Magloire, H., Jurdic, P., and Bleicher, F. (2005). Odontoblast expression of semaphorin 7A during innervation of human dentin. Matrix Biol *24*, 232-238.
- Meister, G., Landthaler, M., Dorsett, Y., and Tuschl, T. (2004). Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. Rna *10*, 544-550.
- Mendelson, B., Koerber, H. R., and Frank, E. (1992). Development of cutaneous and proprioceptive afferent projections in the chick spinal cord. Neurosci Lett *138*, 72-76.
- Messersmith, E. K., Leonardo, E. D., Shatz, C. J., Tessier-Lavigne, M., Goodman, C. S., and Kolodkin, A. L. (1995). Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron *14*, 949-959.
- Mitchell, K. J., Doyle, J. L., Serafini, T., Kennedy, T. E., Tessier-Lavigne, M., Goodman, C. S., and Dickson, B. J. (1996). Genetic analysis of Netrin genes in

Drosophila: Netrins guide CNS commissural axons and peripheral motor axons. Neuron *17*, 203-215.

- Moreau-Fauvarque, C., Kumanogoh, A., Camand, E., Jaillard, C., Barbin, G., Boquet, I., Love, C., Jones, E. Y., Kikutani, H., Lubetzki, C.*, et al.* (2003). The transmembrane semaphorin Sema4D/CD100, an inhibitor of axonal growth, is expressed on oligodendrocytes and upregulated after CNS lesion. J Neurosci *23*, 9229-9239.
- Murakami, Y., Suto, F., Shimizu, M., Shinoda, T., Kameyama, T., and Fujisawa, H. (2001). Differential expression of plexin-A subfamily members in the mouse nervous system. Dev Dyn *220*, 246-258.
- Muramatsu, T., Mizutani, Y., Ohmori, Y., and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. Biochem Biophys Res Commun *230*, 376-380.
- Nakamoto, K., and Shiga, T. (1998). Tissues exhibiting inhibitory [correction of inhibiory] and repulsive activities during the initial stages of neurite outgrowth from the dorsal root ganglion in the chick embryo. Dev Biol *202*, 304-314.
- Nakamura, F., Kalb, R. G., and Strittmatter, S. M. (2000). Molecular basis of semaphorin-mediated axon guidance. J Neurobiol *44*, 219-229.
- Nakamura, F., Tanaka, M., Takahashi, T., Kalb, R. G., and Strittmatter, S. M. (1998). Neuropilin-1 extracellular domains mediate semaphorin D/III-induced growth cone collapse. Neuron *21*, 1093-1100.
- Neufeld, G., Shraga-Heled, N., Lange, T., Guttmann-Raviv, N., Herzog, Y., and Kessler, O. (2005). Semaphorins in cancer. Front Biosci *10*, 751-760.
- Niederlander, C., and Lumsden, A. (1996). Late emigrating neural crest cells migrate specifically to the exit points of cranial branchiomotor nerves. Development *122*, 2367-2374.
- O'Brien, D., Dockery, P., McDermott, K., and Fraher, J. (2001). Early development of rat ventral root transitional zone: an immunohistochemical and morphometric study. J Neurocytol *30*, 11-20.
- O'Brien, D., Dockery, P., McDermott, K., and Fraher, J. P. (1998). The ventral motoneurone axon bundle in the CNS--a cordone system? J Neurocytol *27*, 247-258.
- O'Leary, D. D., and McLaughlin, T. (2005). Mechanisms of retinotopic map development: Ephs, ephrins, and spontaneous correlated retinal activity. Prog Brain Res *147*, 43-65.
- O'Leary, D. D., and Wilkinson, D. G. (1999). Eph receptors and ephrins in neural development. Curr Opin Neurobiol *9*, 65-73.
- Oakley, R. A., and Tosney, K. W. (1991). Peanut agglutinin and chondroitin-6-sulfate are molecular markers for tissues that act as barriers to axon advance in the avian embryo. Dev Biol *147*, 187-206.
- Ohoka, Y., Hirotani, M., Sugimoto, H., Fujioka, S., Furuyama, T., and Inagaki, S. (2001). Semaphorin 4C, a transmembrane semaphorin, [corrected] associates with a

neurite-outgrowth-related protein, SFAP75. Biochem Biophys Res Commun *280*, 237-243.

- Ohta, K., Mizutani, A., Kawakami, A., Murakami, Y., Kasuya, Y., Takagi, S., Tanaka, H., and Fujisawa, H. (1995). Plexin: a novel neuronal cell surface molecule that mediates cell adhesion via a homophilic binding mechanism in the presence of calcium ions. Neuron *14*, 1189-1199.
- Ohta, K., Takagi, S., Asou, H., and Fujisawa, H. (1992). Involvement of neuronal cell surface molecule B2 in the formation of retinal plexiform layers. Neuron *9*, 151-161.
- Oinuma, I., Katoh, H., Harada, A., and Negishi, M. (2003). Direct interaction of Rnd1 with Plexin-B1 regulates PDZ-RhoGEF-mediated Rho activation by Plexin-B1 and induces cell contraction in COS-7 cells. J Biol Chem *278*, 25671-25677.
- Oster, S. F., Bodeker, M. O., He, F., and Sretavan, D. W. (2003). Invariant Sema5A inhibition serves an ensheathing function during optic nerve development. Development *130*, 775-784.
- Ozaki, S., and Snider, W. D. (1997). Initial trajectories of sensory axons toward laminar targets in the developing mouse spinal cord. J Comp Neurol *380*, 215-229.
- Palmer, A., and Klein, R. (2003). Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. Genes Dev *17*, 1429-1450.
- Pasterkamp, R. J., Giger, R. J., and Verhaagen, J. (1998). Regulation of semaphorin III/collapsin-1 gene expression during peripheral nerve regeneration. Exp Neurol *153*, 313-327.
- Pasterkamp, R. J., Peschon, J. J., Spriggs, M. K., and Kolodkin, A. L. (2003). Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. Nature *424*, 398-405.
- Pasterkamp, R. J., Ruitenberg, M. J., and Verhaagen, J. (1999). Semaphorins and their receptors in olfactory axon guidance. Cell Mol Biol (Noisy-le-grand) *45*, 763-779.
- Pasterkamp, R. J., and Verhaagen, J. (2001). Emerging roles for semaphorins in neural regeneration. Brain Res Brain Res Rev *35*, 36-54.
- Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S., and Stoeckli, E. T. (2003). Screening for gene function in chicken embryo using RNAi and electroporation. Nat Biotechnol *21*, 93-96.
- Pfaff, S. L., Mendelsohn, M., Stewart, C. L., Edlund, T., and Jessell, T. M. (1996). Requirement for LIM homeobox gene Isl1 in motor neuron generation reveals a motor neuron-dependent step in interneuron differentiation. Cell *84*, 309-320.
- Placzek, M., Tessier-Lavigne, M., Jessell, T., and Dodd, J. (1990). Orientation of commissural axons in vitro in response to a floor plate-derived chemoattractant. Development *110*, 19-30.
- Polleux, F., Giger, R. J., Ginty, D. D., Kolodkin, A. L., and Ghosh, A. (1998). Patterning of cortical efferent projections by semaphorin-neuropilin interactions. Science *282*, 1904-1906.

- Prasad, A., and Hollyday, M. (1991). Development and migration of avian sympathetic preganglionic neurons. J Comp Neurol *307*, 237-258.
- Puschel, A. W., Adams, R. H., and Betz, H. (1995). Murine semaphorin D/collapsin is a member of a diverse gene family and creates domains inhibitory for axonal extension. Neuron *14*, 941-948.
- Puschel, A. W., Adams, R. H., and Betz, H. (1996). The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. Mol Cell Neurosci *7*, 419-431.
- Qu, X., Wei, H., Zhai, Y., Que, H., Chen, Q., Tang, F., Wu, Y., Xing, G., Zhu, Y., Liu, S.*, et al.* (2002). Identification, characterization, and functional study of the two novel human members of the semaphorin gene family. J Biol Chem *277*, 35574-35585.
- Rabacchi, S. A., Solowska, J. M., Kruk, B., Luo, Y., Raper, J. A., and Baird, D. H. (1999). Collapsin-1/semaphorin-III/D is regulated developmentally in Purkinje cells and collapses pontocerebellar mossy fiber neuronal growth cones. J Neurosci *19*, 4437-4448.
- Rajagopalan, S., Nicolas, E., Vivancos, V., Berger, J., and Dickson, B. J. (2000). Crossing the midline: roles and regulation of Robo receptors. Neuron *28*, 767-777.
- Raper, J. A. (2000). Semaphorins and their receptors in vertebrates and invertebrates. Curr Opin Neurobiol *10*, 88-94.
- Raper, J. A., and Kapfhammer, J. P. (1990). The enrichment of a neuronal growth cone collapsing activity from embryonic chick brain. Neuron *4*, 21-29.
- Renzi, M. J., Feiner, L., Koppel, A. M., and Raper, J. A. (1999). A dominant negative receptor for specific secreted semaphorins is generated by deleting an extracellular domain from neuropilin-1. J Neurosci *19*, 7870-7880.
- Reza, J. N., Gavazzi, I., and Cohen, J. (1999). Neuropilin-1 is expressed on adult mammalian dorsal root ganglion neurons and mediates semaphorin3a/collapsin-1-induced growth cone collapse by small diameter sensory afferents. Mol Cell Neurosci *14*, 317-326.
- Rice, D. S., Huang, W., Jones, H. A., Hansen, G., Ye, G. L., Xu, N., Wilson, E. A., Troughton, K., Vaddi, K., Newton, R. C.*, et al.* (2004). Severe retinal degeneration associated with disruption of semaphorin 4A. Invest Ophthalmol Vis Sci *45*, 2767-2777.
- Rohm, B., Ottemeyer, A., Lohrum, M., and Puschel, A. W. (2000). Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A. Mech Dev *93*, 95-104.
- Rutishauser, U. (2000). Defining a role and mechanism for IgCAM function in vertebrate axon guidance. J Cell Biol *149*, 757-760.
- Sahay, A., Kim, C. H., Sepkuty, J. P., Cho, E., Huganir, R. L., Ginty, D. D., and Kolodkin, A. L. (2005). Secreted semaphorins modulate synaptic transmission in the adult hippocampus. J Neurosci *25*, 3613-3620.

- Sahay, A., Molliver, M. E., Ginty, D. D., and Kolodkin, A. L. (2003). Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. J Neurosci *23*, 6671-6680.
- Sakai, J. A., and Halloran, M. C. (2006). Semaphorin 3d guides laterality of retinal ganglion cell projections in zebrafish. Development.
- Satoda, M., Takagi, S., Ohta, K., Hirata, T., and Fujisawa, H. (1995). Differential expression of two cell surface proteins, neuropilin and plexin, in Xenopus olfactory axon subclasses. J Neurosci *15*, 942-955.
- Scarlato, M., Ara, J., Bannerman, P., Scherer, S., and Pleasure, D. (2003). Induction of neuropilins-1 and -2 and their ligands, Sema3A, Sema3F, and VEGF, during Wallerian degeneration in the peripheral nervous system. Exp Neurol *183*, 489-498.
- Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C., and Charnay, P. (1993). Disruption of Krox-20 results in alteration of rhombomeres 3 and 5 in the developing hindbrain. Cell *75*, 1199-1214.
- Schneider, V. A., and Granato, M. (2003). Motor axon migration: a long way to go. Dev Biol *263*, 1-11.
- Schultze, W., Eulenburg, V., Lessmann, V., Herrmann, L., Dittmar, T., Gundelfinger, E. D., Heumann, R., and Erdmann, K. S. (2001). Semaphorin4F interacts with the synapse-associated protein SAP90/PSD-95. J Neurochem *78*, 482-489.
- Seeger, M., Tear, G., Ferres-Marco, D., and Goodman, C. S. (1993). Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. Neuron *10*, 409-426.
- Serafini, T., Colamarino, S. A., Leonardo, E. D., Wang, H., Beddington, R., Skarnes, W. C., and Tessier-Lavigne, M. (1996). Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. Cell *87*, 1001-1014.
- Serafini, T., Kennedy, T. E., Galko, M. J., Mirzayan, C., Jessell, T. M., and Tessier-Lavigne, M. (1994). The netrins define a family of axon outgrowth-promoting proteins homologous to C. elegans UNC-6. Cell *78*, 409-424.
- Shao, H., Lou, L., Pandey, A., Pasquale, E. B., and Dixit, V. M. (1994). cDNA cloning and characterization of a ligand for the Cek5 receptor protein-tyrosine kinase. J Biol Chem *269*, 26606-26609.
- Shao, H., Lou, L., Pandey, A., Verderame, M. F., Siever, D. A., and Dixit, V. M. (1995). cDNA cloning and characterization of a Cek7 receptor protein-tyrosine kinase ligand that is identical to the ligand (ELF-1) for the Mek-4 and Sek receptor protein-tyrosine kinases. J Biol Chem *270*, 3467-3470.
- Sharma, K., and Frank, E. (1998). Sensory axons are guided by local cues in the developing dorsal spinal cord. Development *125*, 635-643.
- Sharma, K., Sheng, H. Z., Lettieri, K., Li, H., Karavanov, A., Potter, S., Westphal, H., and Pfaff, S. L. (1998). LIM homeodomain factors Lhx3 and Lhx4 assign subtype identities for motor neurons. Cell *95*, 817-828.

- Sharrocks, A. D. (2001). The ETS-domain transcription factor family. Nat Rev Mol Cell Biol *2*, 827-837.
- Shepherd, I., Luo, Y., Raper, J. A., and Chang, S. (1996). The distribution of collapsin-1 mRNA in the developing chick nervous system. Dev Biol *173*, 185-199.
- Shima, D. T., and Mailhos, C. (2000). Vascular developmental biology: getting nervous. Curr Opin Genet Dev *10*, 536-542.
- Shirasaki, R., and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. Annu Rev Neurosci *25*, 251-281.
- Silver, J., and Rutishauser, U. (1984). Guidance of optic axons in vivo by a preformed adhesive pathway on neuroepithelial endfeet. Dev Biol *106*, 485-499.
- Simmer, F., Moorman, C., van der Linden, A. M., Kuijk, E., van den Berghe, P. V., Kamath, R. S., Fraser, A. G., Ahringer, J., and Plasterk, R. H. (2003). Genome-wide RNAi of C. elegans using the hypersensitive rrf-3 strain reveals novel gene functions. PLoS Biol *1*, E12.
- Simmons, A. D., Overhauser, J., and Lovett, M. (1997). Isolation of cDNAs from the Cri-du-chat critical region by direct screening of a chromosome 5-specific cDNA library. Genome Res *7*, 118-127.
- Simmons, A. D., Puschel, A. W., McPherson, J. D., Overhauser, J., and Lovett, M. (1998). Molecular cloning and mapping of human semaphorin F from the Cri-du-chat candidate interval. Biochem Biophys Res Commun *242*, 685-691.
- Sockanathan, S., and Jessell, T. M. (1998). Motor neuron-derived retinoid signaling specifies the subtype identity of spinal motor neurons. Cell *94*, 503-514.
- Sonderegger, P., and Rathjen, F. G. (1992). Regulation of axonal growth in the vertebrate nervous system by interactions between glycoproteins belonging to two subgroups of the immunoglobulin superfamily. J Cell Biol *119*, 1387-1394.
- Steup, A., Ninnemann, O., Savaskan, N. E., Nitsch, R., Puschel, A. W., and Skutella, T. (1999). Semaphorin D acts as a repulsive factor for entorhinal and hippocampal neurons. Eur J Neurosci *11*, 729-734.
- Stoeckli, E. T. (1998). Molecular mechanisms of commissural axon pathfinding. Prog Brain Res *117*, 105-114.
- Stoeckli, E. T., and Landmesser, L. T. (1995). Axonin-1, Nr-CAM, and Ng-CAM play different roles in the in vivo guidance of chick commissural neurons. Neuron *14*, 1165-1179.
- Stoeckli, E. T., and Landmesser, L. T. (1998). Axon guidance at choice points. Curr Opin Neurobiol *8*, 73-79.
- Stoeckli, E. T., Sonderegger, P., Pollerberg, G. E., and Landmesser, L. T. (1997). Interference with axonin-1 and NrCAM interactions unmasks a floor-plate activity inhibitory for commissural axons. Neuron *18*, 209-221.

- Suto, F., Murakami, Y., Nakamura, F., Goshima, Y., and Fujisawa, H. (2003). Identification and characterization of a novel mouse plexin, plexin-A4. Mech Dev *120*, 385-396.
- Swiercz, J. M., Kuner, R., Behrens, J., and Offermanns, S. (2002). Plexin-B1 directly interacts with PDZ-RhoGEF/LARG to regulate RhoA and growth cone morphology. Neuron *35*, 51-63.
- Swiercz, J. M., Kuner, R., and Offermanns, S. (2004). Plexin-B1/RhoGEF-mediated RhoA activation involves the receptor tyrosine kinase ErbB-2. J Cell Biol *165*, 869-880.
- Taillebourg, E., Buart, S., and Charnay, P. (2002). Conditional, floxed allele of the Krox20 gene. Genesis *32*, 112-113.
- Takagi, S., Hirata, T., Agata, K., Mochii, M., Eguchi, G., and Fujisawa, H. (1991). The A5 antigen, a candidate for the neuronal recognition molecule, has homologies to complement components and coagulation factors. Neuron *7*, 295-307.
- Takagi, S., Kasuya, Y., Shimizu, M., Matsuura, T., Tsuboi, M., Kawakami, A., and Fujisawa, H. (1995). Expression of a cell adhesion molecule, neuropilin, in the developing chick nervous system. Dev Biol *170*, 207-222.
- Takahashi, T., Fournier, A., Nakamura, F., Wang, L. H., Murakami, Y., Kalb, R. G., Fujisawa, H., and Strittmatter, S. M. (1999). Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. Cell *99*, 59-69.
- Takahashi, T., Nakamura, F., Jin, Z., Kalb, R. G., and Strittmatter, S. M. (1998). Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. Nat Neurosci *1*, 487-493.
- Takahashi, T., and Strittmatter, S. M. (2001). Plexina1 autoinhibition by the plexin sema domain. Neuron *29*, 429-439.
- Tamagnone, L., Artigiani, S., Chen, H., He, Z., Ming, G. I., Song, H., Chedotal, A., Winberg, M. L., Goodman, C. S., Poo, M.*, et al.* (1999). Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. Cell *99*, 71-80.
- Tamagnone, L., and Comoglio, P. M. (2000). Signalling by semaphorin receptors: cell guidance and beyond. Trends Cell Biol *10*, 377-383.
- Tamagnone, L., and Comoglio, P. M. (2004). To move or not to move? Semaphorin signalling in cell migration. EMBO Rep *5*, 356-361.
- Tanabe, Y., William, C., and Jessell, T. M. (1998). Specification of motor neuron identity by the MNR2 homeodomain protein. Cell *95*, 67-80.
- Tanaka, H. (1991). First step of selective motoneuron axonal growth: selective outgrowth at discrete sites in the spinal cord. J Comp Neurol *303*, 329-337.
- Tang, J., Landmesser, L., and Rutishauser, U. (1992). Polysialic acid influences specific pathfinding by avian motoneurons. Neuron *8*, 1031-1044.

- Tang, J., Rutishauser, U., and Landmesser, L. (1994). Polysialic acid regulates growth cone behavior during sorting of motor axons in the plexus region. Neuron *13*, 405-414.
- Taniguchi, M., Masuda, T., Fukaya, M., Kataoka, H., Mishina, M., Yaginuma, H., Watanabe, M., and Shimizu, T. (2005). Identification and characterization of a novel member of murine semaphorin family. Genes Cells *10*, 785-792.
- Taniguchi, M., and Shimizu, T. (2004). Characterization of a novel member of murine semaphorin family. Biochem Biophys Res Commun *314*, 242-248.
- Taniguchi, M., Yuasa, S., Fujisawa, H., Naruse, I., Saga, S., Mishina, M., and Yagi, T. (1997). Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. Neuron *19*, 519-530.
- Tear, G. (1999). Axon guidance at the central nervous system midline. Cell Mol Life Sci *55*, 1365-1376.
- Tessier-Lavigne, M. (1995). Eph receptor tyrosine kinases, axon repulsion, and the development of topographic maps. Cell *82*, 345-348.
- Tessier-Lavigne, M., and Goodman, C. S. (1996). The molecular biology of axon guidance. Science *274*, 1123-1133.
- Thanos, S., Bonhoeffer, F., and Rutishauser, U. (1984). Fiber-fiber interaction and tectal cues influence the development of the chicken retinotectal projection. Proc Natl Acad Sci U S A *81*, 1906-1910.
- Togari, A., Mogi, M., Arai, M., Yamamoto, S., and Koshihara, Y. (2000). Expression of mRNA for axon guidance molecules, such as semaphorin-III, netrins and neurotrophins, in human osteoblasts and osteoclasts. Brain Res *878*, 204-209.
- Topilko, P., Schneider-Maunoury, S., Levi, G., Baron-Van Evercooren, A., Chennoufi, A. B., Seitanidou, T., Babinet, C., and Charnay, P. (1994). Krox-20 controls myelination in the peripheral nervous system. Nature *371*, 796-799.
- Torres-Vazquez, J., Gitler, A. D., Fraser, S. D., Berk, J. D., Van, N. P., Fishman, M. C., Childs, S., Epstein, J. A., and Weinstein, B. M. (2004). Semaphorin-plexin signaling guides patterning of the developing vasculature. Dev Cell *7*, 117-123.
- Tosney, K. W., Hotary, K. B., and Lance-Jones, C. (1995). Specifying the target identity of motoneurons. Bioessays *17*, 379-382.
- Tosney, K. W., and Landmesser, L. T. (1985a). Development of the major pathways for neurite outgrowth in the chick hindlimb. Dev Biol *109*, 193-214.
- Tosney, K. W., and Landmesser, L. T. (1985b). Specificity of early motoneuron growth cone outgrowth in the chick embryo. J Neurosci *5*, 2336-2344.
- Tosney, K. W., and Oakley, R. A. (1990). The perinotochordal mesenchyme acts as a barrier to axon advance in the chick embryo: implications for a general mechanism of axonal guidance. Exp Neurol *109*, 75-89.
- Toyofuku, T., Zhang, H., Kumanogoh, A., Takegahara, N., Suto, F., Kamei, J., Aoki, K., Yabuki, M., Hori, M., Fujisawa, H., and Kikutani, H. (2004a). Dual roles of

Sema6D in cardiac morphogenesis through region-specific association of its receptor, Plexin-A1, with off-track and vascular endothelial growth factor receptor type 2. Genes Dev *18*, 435-447.

- Toyofuku, T., Zhang, H., Kumanogoh, A., Takegahara, N., Yabuki, M., Harada, K., Hori, M., and Kikutani, H. (2004b). Guidance of myocardial patterning in cardiac development by Sema6D reverse signalling. Nat Cell Biol *6*, 1204-1211.
- Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M., and Pfaff, S. L. (1994). Topographic organization of embryonic motor neurons defined by expression of LIM homeobox genes. Cell *79*, 957-970.
- Ulupinar, E., Datwani, A., Behar, O., Fujisawa, H., and Erzurumlu, R. (1999). Role of semaphorin III in the developing rodent trigeminal system. Mol Cell Neurosci *13*, 281-292.
- Usui, H., Taniguchi, M., Yokomizo, T., and Shimizu, T. (2003). Plexin-A1 and plexin-B1 specifically interact at their cytoplasmic domains. Biochem Biophys Res Commun *300*, 927-931.
- van der Zwaag, B., Hellemons, A. J., Leenders, W. P., Burbach, J. P., Brunner, H. G., Padberg, G. W., and Van Bokhoven, H. (2002). PLEXIN-D1, a novel plexin family member, is expressed in vascular endothelium and the central nervous system during mouse embryogenesis. Dev Dyn *225*, 336-343.
- Varela-Echavarria, A., and Guthrie, S. (1997). Molecules making waves in axon guidance. Genes Dev *11*, 545-557.
- Varela-Echavarria, A., Tucker, A., Puschel, A. W., and Guthrie, S. (1997). Motor axon subpopulations respond differentially to the chemorepellents netrin-1 and semaphorin D. Neuron *18*, 193-207.
- Vermeren, M., Maro, G. S., Bron, R., McGonnell, I. M., Charnay, P., Topilko, P., and Cohen, J. (2003). Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points. Neuron *37*, 403-415.
- Vikis, H. G., Li, W., and Guan, K. L. (2002). The plexin-B1/Rac interaction inhibits PAK activation and enhances Sema4D ligand binding. Genes Dev *16*, 836-845.
- Vikis, H. G., Li, W., He, Z., and Guan, K. L. (2000). The semaphorin receptor plexin-B1 specifically interacts with active Rac in a ligand-dependent manner. Proc Natl Acad Sci U S A *97*, 12457-12462.
- Voiculescu, O., Charnay, P., and Schneider-Maunoury, S. (2000). Expression pattern of a Krox-20/Cre knock-in allele in the developing hindbrain, bones, and peripheral nervous system. Genesis *26*, 123-126.
- Voiculescu, O., Taillebourg, E., Pujades, C., Kress, C., Buart, S., Charnay, P., and Schneider-Maunoury, S. (2001). Hindbrain patterning: Krox20 couples segmentation and specification of regional identity. Development *128*, 4967-4978.

- Wang, G., and Scott, S. A. (2000). The "waiting period" of sensory and motor axons in early chick hindlimb: its role in axon pathfinding and neuronal maturation. J Neurosci *20*, 5358-5366.
- Wang, L., Zeng, H., Wang, P., Soker, S., and Mukhopadhyay, D. (2003). Neuropilin-1-mediated vascular permeability factor/vascular endothelial growth factor-dependent endothelial cell migration. J Biol Chem *278*, 48848-48860.
- Watanabe, Y., Toyoda, R., and Nakamura, H. (2004). Navigation of trochlear motor axons along the midbrain-hindbrain boundary by neuropilin 2. Development *131*, 681-692.
- White, F. A., and Behar, O. (2000). The development and subsequent elimination of aberrant peripheral axon projections in Semaphorin3A null mutant mice. Dev Biol *225*, 79-86.
- Wilkinson, D. G. (2000). Eph receptors and ephrins: regulators of guidance and assembly. Int Rev Cytol *196*, 177-244.
- Wilkinson, D. G., Bhatt, S., Chavrier, P., Bravo, R., and Charnay, P. (1989). Segment-specific expression of a zinc-finger gene in the developing nervous system of the mouse. Nature *337*, 461-464.
- Williams-Hogarth, L. C., Puche, A. C., Torrey, C., Cai, X., Song, I., Kolodkin, A. L., Shipley, M. T., and Ronnett, G. V. (2000). Expression of semaphorins in developing and regenerating olfactory epithelium. J Comp Neurol *423*, 565-578.
- Winberg, M. L., Noordermeer, J. N., Tamagnone, L., Comoglio, P. M., Spriggs, M. K., Tessier-Lavigne, M., and Goodman, C. S. (1998). Plexin A is a neuronal semaphorin receptor that controls axon guidance. Cell *95*, 903-916.
- Wright, D. E., White, F. A., Gerfen, R. W., Silos-Santiago, I., and Snider, W. D. (1995). The guidance molecule semaphorin III is expressed in regions of spinal cord and periphery avoided by growing sensory axons. J Comp Neurol *361*, 321-333.
- Xiang, R. H., Hensel, C. H., Garcia, D. K., Carlson, H. C., Kok, K., Daly, M. C., Kerbacher, K., van den Berg, A., Veldhuis, P., Buys, C. H., and Naylor, S. L. (1996). Isolation of the human semaphorin III/F gene (SEMA3F) at chromosome 3p21, a region deleted in lung cancer. Genomics *32*, 39-48.
- Xiao, T., Shoji, W., Zhou, W., Su, F., and Kuwada, J. Y. (2003). Transmembrane sema4E guides branchiomotor axons to their targets in zebrafish. J Neurosci *23*, 4190-4198.
- Xu, X., Ng, S., Wu, Z. L., Nguyen, D., Homburger, S., Seidel-Dugan, C., Ebens, A., and Luo, Y. (1998). Human semaphorin K1 is glycosylphosphatidylinositol-linked and defines a new subfamily of viral-related semaphorins. J Biol Chem *273*, 22428-22434.
- Xu, X. M., Fisher, D. A., Zhou, L., White, F. A., Ng, S., Snider, W. D., and Luo, Y. (2000). The transmembrane protein semaphorin 6A repels embryonic sympathetic axons. J Neurosci *20*, 2638-2648.

- Yin, Y., Sanes, J. R., and Miner, J. H. (2000). Identification and expression of mouse netrin-4. Mech Dev *96*, 115-119.
- Yu, H. H., and Moens, C. B. (2005). Semaphorin signaling guides cranial neural crest cell migration in zebrafish. Dev Biol *280*, 373-385.
- Yu, T. W., and Bargmann, C. I. (2001). Dynamic regulation of axon guidance. Nat Neurosci *4 Suppl*, 1169-1176.
- Yukawa, K., Tanaka, T., Bai, T., Ueyama, T., Owada-Makabe, K., Tsubota, Y., Maeda, M., Suzuki, K., Kikutani, H., and Kumanogoh, A. (2005). Semaphorin 4A induces growth cone collapse of hippocampal neurons in a Rho/Rho-kinase-dependent manner. Int J Mol Med *16*, 115-118.
- Zhou, L., White, F. A., Lentz, S. I., Wright, D. E., Fisher, D. A., and Snider, W. D. (1997). Cloning and expression of a novel murine semaphorin with structural similarity to insect semaphorin I. Mol Cell Neurosci *9*, 26-41.
- Zou, Y., Stoeckli, E., Chen, H., and Tessier-Lavigne, M. (2000). Squeezing axons out of the gray matter: a role for slit and semaphorin proteins from midline and ventral spinal cord. Cell *102*, 363-375.

Chapter :6

CURRICULUM VITAE

SURNAME:	SADHU	**FIRST NAME:**	REJINA
DATE OF BIRTH:	5TH APRIL 1975	**NATIONALITY**:	INDIAN

ACADEMIC PROFILE

1992-1995: **Bachelor's Degree in Zoology and Biochemistry**, University of Mumbai, India

1996-2001: **Master's Degree by Research in Applied Biology**, University of Mumbai, India
Master's Thesis: 'Organ specific safety of turmeric oil and other medicinal properties'

2001-2006: **Ph.D. in Developmental Neurobiology,**
Institute of Zoology, University of Zurich (Supervisor: Prof. Esther T. Stoeckli).

AWARDS / HONOURS:

- Master's thesis recommended for prolongation into Ph.D. (2001).
- Article on Fullerenes selected for special appreciation by Prof.Harold Kroto (Nobel Laureate) and Prof. D.R.M Walton, University of Sussex (1994)
- Invited to speak at the first State Level Conference of the Indian Oral Health Education and Research Centre (IOHERC) on 'Turmeric and Chemoprevention: An Overview on Oral Cancer' in Mumbai, India (2001)

LIST OF PUBLICATIONS

- ***In ovo* RNAi opens new possibilities for functional genomics in vertebrates.** Dimitris Bourikas, Thomas Baeriswyl, Rejina Sadhu, and Esther T. Stoeckli. Chapter in a book titled: **'RNA Interference Technology- From Basic Science to Development'** edited by Krishnarao Appasani, Cambridge University Press, First published in February 2005.

- **Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord.** Dimitris Bourikas, Vladimir Pekarik, Thomas Baeriswyl, Åsa Grunditz, Rejina Sadhu, Michele Nardó & Esther T Stoeckli. **Nature Neuroscience**, Vol.8 (Kirk and Allen) 297-304, March 2005.
- Expression patterns of plexins and neuropilins suggest cooperative and separate functions in spinal cord development. Olivier Mauti, Rejina Sadhu, Joelle Gemayel, Matthias Gesemann and Esther T. Stoeckli. (Submitted).
- Combinatorial expression of semaphorins in chicken motor and sensory neurons suggests alternative semaphorin functions in axon growth and guidance. Joelle Gemayel, Pascal Joset, Olivier Mauti, Rejina Sadhu, Esther Stoeckli and Matthias Gesemann. (Submitted).

Chapter: 7

ACKNOWLEDGEMENTS

- I am thankful to Prof. Esther T. Stoeckli for giving me the opportunity to work towards my Ph.D. in her lab. Her enthusiasm for science and readiness to answer all queries has always been a source of motivation to me. Her patience, kindness and support through my Ph.D. are unforgettable. I have learnt a lot in these years. The experience was full of many a flavor and hue.
- I am grateful to Prof. Martin Schwab for being very supportive and encouraging throughout my Ph.D. His knowledge of neuroscience and zest for living has been a constant inspiration to me.
- I am thankful to Prof. Alex Hajnal, Dr. Matthias Gesemann and Prof. Alain Chedotal for agreeing to be on my Ph.D. Thesis committee. Special thanks to Matthias and Alex for all their valuable comments during the shaping of the project.
- I am indebted to all my ex-colleagues (Dr. Vladimir Pekarik, Dr. Melanie Philipp, Dr. Stephanie Albert, Dr. Beatrix Otte and Monika Mielich) for teaching me the ropes in my Ph.D.
- I am thankful to Dimitris Bourikas and Thomas Baeriswyl for being the most wonderful colleagues one can ever have. Supervising talented students like Olivier Mauti and Anna Lena Daetwyler has been a delightful experience.
- Our lab would not have been the delightful place it was if we did not have our other lab members (ex- and present): Åsa Grunditz, Michele Nardo, Larissa Vines, Pascal Joset, Stephan Preiswerk, Richard Prazak, Marc Debrunner, Andrin Wacker, Claudia Litscher, Maja Hess, Irwin Andermatt, Elena Domanitskaya and Vera Niederkofler.
- Memorable moments with Hai Qiaong, Beatrix Otte, Seraina Keller and Stephanie Albert made my first time in a foreign country very pleasant. Words are not enough to express my thankfulness for the support and friendship of two lovely people -Ronja Bahadori and Joelle el Gemayel. Thanks for all those lovely times that we shared.

- My friends from Basel, Zurich, Dietikon, Fribourg and St.Gallen made Switzerland (and the ones who left Switzerland for U.S.) feel like home. Their friendship and fun-loving nature was pure pleasure. With them around, India did not seem so far away.
- Across miles, through thick and thin, I have had the friendship of Aditi, Anupama, Hetal, Jyotsna, Neelambari, Sonal and Vidya. Thanks for being there for me.
- Words cannot express my gratitude and love for my parents and brother for believing in me.
- I am thankful to my parents-in-law for being supportive throughout my Ph.D. Thanks to Poly and Soumya for being the people you are.
- Last but not the least I am thankful to my dearest husband and best friend- Sudip for being there for me at every step. His encouragement and confidence in me has kept me going through very trying times.
- There are many people known and unknown who with their smiles, kindness, determination and compassion have touched my life. Thanks to all of them and to Almighty for giving me the strength and confidence to go ahead in life.